JOURNAL OF THE KENTUCKY ACADEMY OF SCIENCE

Official Publication of the Academy

Volume 66
Number 2
Fall 2005

Volume 66 of the *Journal of the Kentucky Academy of Science*

is dedicated to the memory of

John W. Thieret, Editor (1996–2005)

JOHN W. THIERET (1926–2005)

JOURNAL OF THE KENTUCKY ACADEMY OF SCIENCE
ISSN 1098-7096
Continuation of
Transactions of the Kentucky Academy of Science
Volume 66 Fall 2005 Number 2

J. Ky. Acad. Sci. 66(2):73–81. 2005.

John W. Thieret (1926–2005)

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ABSTRACT

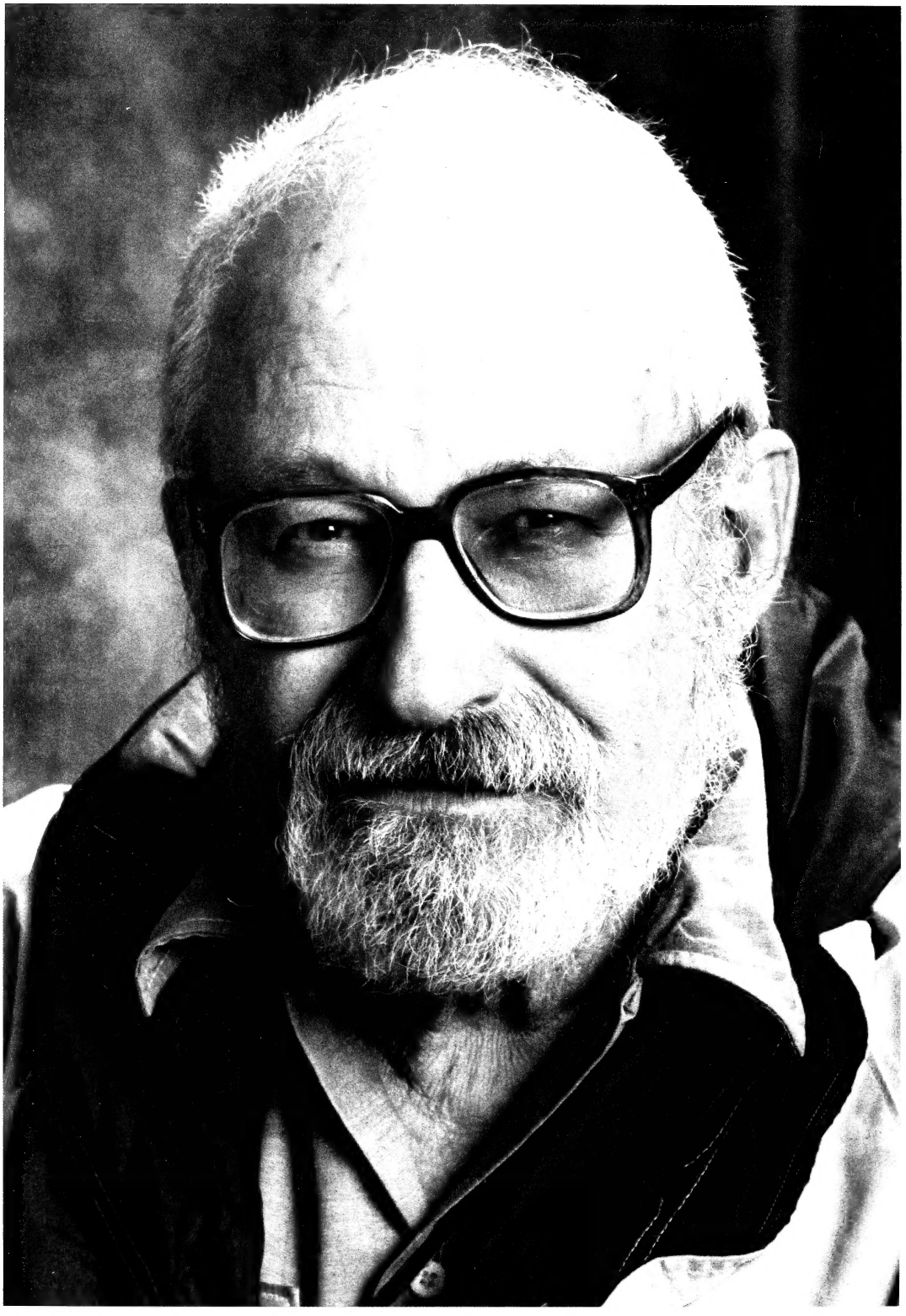
John W. Thieret (1926–2005), an internationally recognized American plant taxonomist, is remembered as a consummate field botanist, exemplary teacher, acclaimed author, superb editor, fine herbarium director, inspiring mentor to students and colleagues, and noble friend.

One of the most renowned American plant taxonomists of the 20th century has died. It is with great sadness and a profound sense of loss that I write this tribute about the life and career of John W. Thieret, Professor Emeritus of Biological Sciences at Northern Kentucky University, retired Director of the Northern Kentucky University Herbarium, and Editor of the *Journal of the Kentucky Academy of Science* (JKAS). John suffered a brain aneurysm at his home in Alexandria, Kentucky, on 6 December 2005, while editing a manuscript for the JKAS. He never regained consciousness and died on 7 December at the age of 79.

John Thieret was a gifted botanist, excellent educator, wise scholar, and gentleman. He dearly loved his family, and after family, his great passion was plants. Although John was focused on botany his entire life, he had other diverse interests including classical music, particularly opera. He also enjoyed sharing his knowledge far beyond botany and the natural sciences through verbal discourse about diverse topics in history, literature, art, religion, and the social sciences.

John William Thieret was born on 1 August 1926, in Chicago, Illinois, the only child of Hans and Lorena Thieret. Growing up, he was interested in plants and became an avid student of botany during his school days at Hyde Park High School. At Hyde Park, John met his future wife, Mildred Wolf, fittingly in a botany class. After working briefly in Chicago, John moved to Logan, Utah, to study at Utah State University. Three years later, Mildred also moved to Logan and attended Utah State. They were married on 13 March 1950, by one of their professors, after completing their Evolution final exams. Both earned their B.S. degrees in 1950: John's in Botany and Mildred's in Bacteriology. They remained at Utah State University for graduate work and in 1951, John earned his M.S. in Botany with focus on barley genetics and Mildred completed her M.S. in Bacteriology. They returned to Chicago, where John attended the University of Chicago to work on his doctorate under Theodor K. Just, Chief Curator of the Chicago Field Museum of Natural History. John received his Ph.D. in Botany in 1953. The title of John's dissertation was "Gross Morphology of the Seeds of the Scrophulariaceae and Classification of the Family."

Later in 1953, John became Assistant Curator of Economic Botany at the Chicago Field Museum and then Curator of Economic Botany from 1954 to 1961. While at the Field



Museum, he made collecting trips to Cuba, Mexico, the northern Great Plains of the United States, and the Northwest Territories of Canada. John authored 26 publications during that time and his special interests in the Poaceae and Scrophulariaceae were evident in his published works. John published five new nomenclatural combinations while at the Field Museum. He also wrote three articles on the flora and vegetation of the Canadian Northwest Territories.

John left the Field Museum to become Associate Professor and later Professor of Biology at the University of Southwestern Louisiana, Lafayette (USL), from 1961 to 1973. At USL, he sponsored undergraduate research projects and directed six M.S. theses and one Ph.D. dissertation. During this period, John conducted most of his personal research in the southeastern United States, with an emphasis on the Louisiana flora. At USL, he authored or co-authored 47 articles. John named four plant species new to science that he discovered in Louisiana: *Cyperus brevifolioides* Thieret & Delahoussaye; *Cyperus louisianensis* Thieret; *Isoetes louisianensis* Thieret; and *Limnophila* ×*ludoviciana* Thieret. From his work at USL, John published 11 nomenclatural combinations. He also published his first two generic flora treatments of the southeastern United States, five more articles from the Canadian Northwest Territories, and several North American plant records.

John made his final academic career move in 1973, when he joined the faculty at Northern Kentucky University (NKU) in Highland Heights, as Professor and Chair of the Department of Biological Sciences. The opportunity to teach at NKU, the presence of the Lloyd Library in Cincinnati, and relocating his family to live in a cooler climate, were especially appealing to him. John served as Chair until 1980 and continued as Professor until retiring in 1992, with the title Professor Emeritus of Biological Sciences.

John loved teaching and was an exemplary teacher in the classroom, laboratory, and field. His classes were challenging, enjoyable, informative, popular, and inspired many students toward their full potential. He always was available to students and colleagues for discussions about botany, academics, or just about life. John was a strong believer in providing students with actual plant specimens, either fresh or dried, for a "hands-on" approach, and he went to great effort to accomplish that goal. In the field, John's passion for botany was especially contagious and he always felt rejuvenated after a field trip with students.

At NKU, John taught 15 different courses for the Department of Biological Sciences including Agrostology, Aquatic Vascular Plants, Dendrology, Field Botany, General Botany, General Biology Lab, Horticultural Plants, Library Resources in Biology, Plants and People, Plants in Winter, Plant Taxonomy, Spring Flora of Kentucky, Summer Flora of Kentucky, Trees of Kentucky, and Woody Plants. John generously shared his expertise and enthusiasm for botany, especially plant taxonomy, with students, colleagues, and the general public for 32 years. His exuberance, magnetic personality, and unparalleled depth and breadth of knowledge were inspirations to everyone his activities touched.

For many years, John's summer activities were focused on teaching at various biological field stations. He served as Visiting Lecturer in Botany at the Itasca Biological Station, University of Minnesota; the Oklahoma Biological Station, University of Oklahoma; the Michigan Biological Station, University of Michigan; and the Franz Theodore Stone Laboratory, Ohio State University. John enjoyed the flora of cooler climates, and biological stations provided Mildred and his active children (Robert, Nancy, Richard, Jeffrey, and Jennifer) new environments to experience. He also conducted major field travels in the southeastern, southwestern, and northwestern United States, the Great Basin of Nevada and Utah, and the Canadian Arctic, Newfoundland, and Ontario.

Scientists are often judged by the number of articles by them or about them. If this is any measure of a person, John stands taller than a coastal redwood. During his professional career, John authored at least 157 refereed journal articles and book articles. Many of his articles appeared in *Sida, Contributions to Botany.* He also published in *Bartonia, Canadian Field-Naturalist, Castanea, Economic Botany, Journal of the Arnold Arboretum, Rhodora, Taxon,* and the *Transactions/Journal of the Kentucky Academy of Science.* In addition to books, journal articles, and 19 no-

menclatural combinations, John wrote 136 book reviews, 65 articles for *Encyclopaedia Britannica,* 46 articles for *Encyclopedia Americana,* and many popular science articles.

During his tenure at NKU, John was the author or co-author of five books. His books were entitled: *Louisiana Ferns and Fern Allies*; *Aquatic and Wetland Plants of Kentucky*; *Trees: A Quick Reference Guide to Trees of North America*; *Assessment and Management of Plant Invasions*; and *National Audubon Society Field Guide to North American Wildflowers: Eastern Region.* His books continue to serve many audiences, from professional botanists to amateur plant enthusiasts.

John authored or co-authored with other botanists, 58 refereed journal articles at NKU. He frequently collaborated with younger NKU biology colleagues in research activities and publications. John also generously shared his research interests with current and former undergraduate and graduate students. He did not restrict his research activities to colleagues and students. Indeed, John was a general mentor for botanists from other institutions to pursue research activities in Kentucky and elsewhere.

His collaborative research with Kentucky botanists ranged from the 150-year chronology of Amur honeysuckle (*Lonicera maackii*) and people, to the history of the medlar (*Mespilus germanica*), to the invasive spread of *Coincya* (*Coincya monensis*) in the eastern United States, to "coffee" from the Kentucky coffeetree, to new Kentucky plant distribution records from two of his favorite weedy haunts, Silver Grove Railroad Yard in Campbell County and Latonia Railroad Yard in Kenton County.

In the 1980s, John initiated an effort with other Kentucky botanists to produce a manual of the Kentucky flora. Several published contributions to that effort resulted, but at a rate too slow to produce a complete state flora. In the mid-1990s, inspired by John's continual encouragement and editorial assistance, Ronald L. Jones of Eastern Kentucky University became the author of the Kentucky flora, with other botanists contributing selected group treatments. As Editorial Associate, John worked closely with Ronald over the next decade by editing many drafts of the manuscript and contributing the treatment of the grasses. In 2005, Jones' comprehensive book, *Plant Life of Kentucky,* was published.

One of John Thieret's crowning achievements was establishing the Northern Kentucky University Herbarium (KNK) in 1973. As the first Director of the KNK Herbarium, he built the herbarium from his personal collections, additions by colleagues and students, and through an active specimen exchange with various national and international herbaria. Currently, KNK has over 35,000 mounted specimens with strong representation from Kentucky, the Southeast, and the Midwest. John's career specimen accession numbers were over 62,000. John accurately identified all plants deposited into the herbarium and meticulously mounted his own plant specimens. The KNK collection has the highest species diversity among Kentucky herbaria collections. Because of his efforts, the KNK herbarium is also the best-curated herbarium in Kentucky. John donated his reference library (over 600 books) to the herbarium.

John served several significant editorships and advisory roles during his professional career. He was a Member of the Editorial Board of *Economic Botany* from 1959–1965, Book Editor of *Economic Botany* from 1959–1984, Editor from 1986–1990, and Associate Editor from 1992–2005. He was a founding Member of the Editorial Board for the Vascular Flora of the Southeastern United States project from 1981–2005. John served as the Associate Editor of *Sida, Contributions to Botany,* from 1971–2005, and contributed to its excellence and prestige through dedicated work. Barney L. Lipscomb, current Editor of *Sida,* Botanical Research Institute of Texas, notes, "John was a towering lighthouse to the editors of *Sida.* His steadfast editorial 'light' was a never ending source of guidance and navigation in keeping *Sida* on a positive course."

During 1983–2005, one of John's most significant roles was as a Member of the Editorial Committee of the monumental multi-volume project, *Flora of North America North of Mexico.* John edited the first 10 published volumes and prepared 25 family and generic treatments. He also was an Advisor in Botany for *Encyclopaedia Britannica* from 1959–2005 and a Member of the Advisory Committee at Lloyd Library in Cincinnati from 1992–2005.

John loved editing and his ability as an ed-

itor was extraordinary. He was a perfectionist and a superlative editor second to none, but his efforts always brought out the best in authors. Those who submitted manuscripts for John's editorial scrutiny often found them returned with a profusion of red ink on the printed text. When his recommended changes were made, the greatly improved manuscripts always told the story better.

Many Kentuckians may best remember John for his devoted service to the Kentucky Academy of Science (KAS). He served as Abstract Editor of *Transactions of the Kentucky Academy of Science* (TKAS) from 1981–1995. John then assumed the position of Editor of TKAS in 1996. He had the title changed from *Transactions* to *Journal of the Kentucky Academy of Science* (JKAS) in 1998 to better reflect its mission and content. Under John's editorship, all JKAS manuscripts were peer-reviewed to meet the highest standards. Since John worked without assistance, he was entirely responsible for all organizational aspects of each JKAS issue. The stature of the JKAS as a multi-disciplinary journal of state and regional scientific literature was greatly enhanced because of his dedicated efforts.

John received many awards and honors during his career. One of his most esteemed honors was having a mint in the Lamiaceae that he discovered in Louisiana named for him. In 1964, botanist Lloyd H. Shinners named this new species *Scutellaria thieretii* Shinners.

John received the 1984 Distinguished Kentucky College/University Scientist Award from the Kentucky Academy of Science for his significant academic research and teaching contributions to the Commonwealth. Most recently, John was presented the 2005 Outstanding Academy Service Award from the Kentucky Academy of Science for his outstanding editorial contributions to the JKAS.

To commemorate his contributions to the Northern Kentucky University Herbarium, it was officially renamed the John W. Thieret Herbarium by the Northern Kentucky University Board of Regents on 22 March 2006.

In 1994, The John W. Thieret Research Award was established by John as an annual award to the NKU student who accomplished the most significant research. The Thieret family would like to continue this student honor, and have asked that donations in his memory be sent to the Northern Kentucky University Foundation, designated to that award.

John is survived by his devoted wife of 55 years, Mildred Thieret, his five children, Robert, Nancy, and Jeffrey in Minnesota, Richard in China, and Jennifer in Highland Heights, seven grandchildren, and five great-grandchildren.

John Thieret was one of the patriarchs in North American plant taxonomy, and one of the last great field naturalists. His death marks the ending of a botanical era, but his legacy continues through the work of many former students and colleagues. John was an inspiring and stabilizing mentor who enriched the lives of those who knew him. He will be greatly missed by all.

ACKNOWLEDGMENTS

I am especially grateful to Mildred Thieret for graciously sharing her memories about John and providing photographs of her husband. I also acknowledge contributions from Ronald L. Jones, Eastern Kentucky University, David M. Brandenburg, Dawes Arboretum, James O. Luken, Coastal Carolina University, Debra K. Pearce, Northern Kentucky University, and Barney L. Lipscomb, Botanical Research Institute of Texas.

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J. Ky. Acad. Sci. 66(2):82–88. 2005.

Cepaea nemoralis (Gastropoda, Helicidae): The Invited Invader

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ABSTRACT

Marauding snails may not immediately come to mind when considering invasive species, but many nonnative snails have successfully colonized the U.S. The wood snail, *Cepaea nemoralis* (L.), is one of the most striking of these introductions, due in part to its attractive shell coloration. This is one of the few snails that people have purposely introduced into their gardens. Several populations are now established in Kentucky, including a newly discovered population in Kenton County. The bright, striped or solid, yellow, pink, and brown shells of this species have long caught the eyes of natural historians and biologists. Populations of these snails are classic model systems for ecological genetics studies. While introduced populations of wood snails seem to have had only minor impact as agricultural pests, they may have the potential to competitively exclude some native species of snails.

INTRODUCTION

Though snails may not be the speediest of beasts, several species have launched successful invasions of the U.S. (Cowie and Robinson 2001; Dundee 1974; Mead 1971). Perhaps the most glamorous of these invaders is the wood snail, *Cepaea nemoralis* (L.). While many gastropods rely on stealth and interstate shipping to fuel their spread, wood snails have another weapon in their arsenals: charm. Their colorful shells (see Figure 1) are nearly irresistible to small children, nostalgic malacologists, and many an evolutionary biologist.

TAXONOMY

The wood snail belongs to the family Helicidae, which includes the bulk of the European edible snails. It is a Linnaean species described in 1758 and was originally *Helix nemoralis,* until Held established the genus *Cepaea* in 1837 (Abbott 1989). Currently, four species are included in the genus, of which *C. nemoralis* is the type. The specific epithet means 'of the woods' or 'inhabiting woods/groves' (Pilsbry 1939; Reeve 1863; Rimmer 1907).

Cepaea hortensis, the white-lipped grove snail, is considered the sister species of *C. nemoralis* (Jones et al. 1977). Historically, taxonomists often treated *C. hortensis* as a variant of *C. nemoralis* (Step 1901). The two species are primarily differentiated by lip color of the shells, which seems a minor feature in light of the fact that *C. nemoralis* is the most variably colored species in its genus and perhaps even among European land snails. However, Rimmer (1907) argued in support of recognizing *C. hortensis,* having observed several mixed populations and noting that of the many snails seen paired on tree trunks, he saw no "matrimonial alliances between these two forms." Current taxonomists also take this view, and the occasional hybrids produced by these species are sterile (Jones et al. 1977). Both taxa occur in the U.S., and though wood snails are known to be introduced, there is disagreement on whether *C. hortensis* is native or was also introduced from Europe (Burch 1962; Dundee 1974; Jones et al. 1977; Mead 1971).

LIFE HISTORY AND NATIVE RANGE

Native to central and western Europe, wood snails are widespread in disturbed habitats, from woodlands to fields and yards, but are also found on chalk cliffs and even coastal dunes (Reeve 1863). They are known by a variety of common names, the English ones including banded grove snail, banded wood snail, brown-lipped snail, and girdled snail (Abbott 1989; Reed 1964; Step 1901; Turton 1857). This species has been widely introduced and now has a nearly worldwide distribution (Abbott 1989).

Wood snails are obligately outcrossing hermaphrodites, with both individuals exchanging sperm during mating, and both individuals able to lay eggs afterward (Stine 1989). Like other members of the Helicidae, *Cepaea* snails have a bizarre courtship behavior in which the courting pair stabs each other with sharp, calcareous structures, aptly named darts, before mating (Abbott 1989; Pilsbry 1939). Wood

Figure 1. Shells of *Cepaea nemoralis* (L.), the wood snail, showing solid pink (left), solid yellow (top), and striped morphs. Note the brown lip characteristic of this species. These European snails have been introduced throughout the northeastern U.S. and occur in at least three counties of Kentucky.

snails often mate multiple times prior to egg laying and can store sperm for up to 15 months (Murray 1964). It is not unusual for one clutch of eggs to include offspring from two different fathers (Murray 1964). Eggs are buried in moist soil, hatching after about 3 weeks (Abbott 1989). The snails reach maturity in 4 years and may live as long as 5–9 years (Abbott 1989; Jones et al. 1977).

Like those of most land snails, wood snail shells are dextral (spiraling to the right), though rare sinistral individuals are sometimes seen (Rimmer 1907; Turton 1857). Mature individuals of *C. nemoralis* reach 2–2.5 cm in diameter and have five whorls to the shell (Pilsbry 1939). When the snail reaches full size and ceases to grow, a reflexed lip forms around the aperture of the shell. The dark brown coloration of this lip differentiates *C. nemoralis* from the similar *C. hortensis*. Shell color varies from yellow, to pinkish, brown, or occasionally even white (Step 1901; Turton 1857). Shells are also generally augmented with 1–5 dark brown bands, though unstriped shells are seen as well (Figure 2). Multiple color variants are commonly found within the same population.

Wood snails, feeding primarily at night, eat a variety of plants, though they often prefer dead plant material to living, and may even forage on dead organisms such as worms or other snails (Thompson 1996; Turton 1857). Among living plant materials, they prefer broad-leaved plants over the tougher grasses

Figure 2. Typical five-striped wood snail morph. There is such great variation in shell striping for this species that a coding system has been developed to catalogue it (Howe 1898; Jones et al. 1977). Stripes are numbered from top to bottom. This shell would be coded 12345. A shell with only the third stripe present (Figure 3) would be 00300. When partial or total fusion of bands is taken into account, there are hundreds of possible variants.

which are common in their habitats, and avoid species with high concentrations of secondary compounds or physical defenses against herbivory such as hairs (Grime et al. 1968; Thompson 1996). Oddly enough, they are said to particularly favor the leaves of stinging nettles (Step 1901). They are adaptable in the lab or under cultivation and happily eat lettuce, carrots, fruit, pure cellulose filter paper, and even (occasionally) mutton (Grime 1968; Judd 1953; Murray 1964; Sowerby 1825; Thompson 1996).

Though these snails lack operculums which would allow them to close their shells, they have a relatively high drought tolerance due to their ability to aestivate. After feeding, they generally crawl up onto the plant or a nearby shrub or stone wall, stick themselves down with a dab of slime, and remain inactive until the next moist evening. During dry spells, these organisms can remain dormant for long periods of time until conditions improve. (Aestivation in many species of snails can last for months, and even for years in some [Abbott 1989].) For the winter months, the snails bury into the soil and remain dormant until spring (Lovell 1884).

In Great Britain, song thrushes can be a major predator of adult wood snails, crushing their shells on stones to get at the soft snail within. Other birds, including chickens, will sometimes eat wood snails (Howe 1898). Several snail predators are invertebrate organisms, including certain beetles, glowworm larvae (related to fireflies), and even predatory snails (Jones et al. 1977; Woodward 1913). Small mammals such as shrews, moles, and hedgehogs also enjoy these slow-moving morsels (Dees 1970; Reed 1964; Woodward 1913).

SPREAD IN THE U.S.

Since the arrival of Europeans, many species of molluscs have been both purposely and accidentally introduced into the U.S. In recent years, the number one pathway for the introduction of new land snail species seems to be via infested horticultural materials (Cowie and Robinson 2001). Eggs and small individuals such as juveniles can be difficult to see when intermixed with soil, mulch, or other plant material (Cowie and Robinson 2001). Many snail species can also self-fertilize or store sperm for up to a year after mating, so one overlooked adult may be all it takes to pioneer an invasion (Cowie and Robinson 2001; Thompson 1996). Wood snails specifically have also been found by the USDA stuck to vehicles and military cargo (Dundee 1974).

The helicid snails, which include *C. nemoralis,* have a somewhat more colorful history of introduction, as many of these were purposely established in new habitats (Dees 1970; Mead 1971). Helicids, such as *Helix pomatia,* the French escargot, are often prized as choice comestibles. Many helicid introductions can be traced back to the kitchen gardens of European immigrants desiring a renewable source of snails (Mead 1971).

As a small and unusually colorful species, *C. nemoralis* has the distinction of being more often introduced for ornament than for food. The earliest U.S. introduction of this species was made by malacologist William Binney in 1857 (Pilsbry 1939). Binney collected snails in Sheffield, England, returned to the U.S. and then released them in his Burlington, NJ, garden, where they proceeded to flourish (Binney and Bland 1869).

The U.S. populations of *C. nemoralis* originate from multiple sources, however. A Lexington, VA, population was attributed either to an introduction of Italian snails in packing ma-

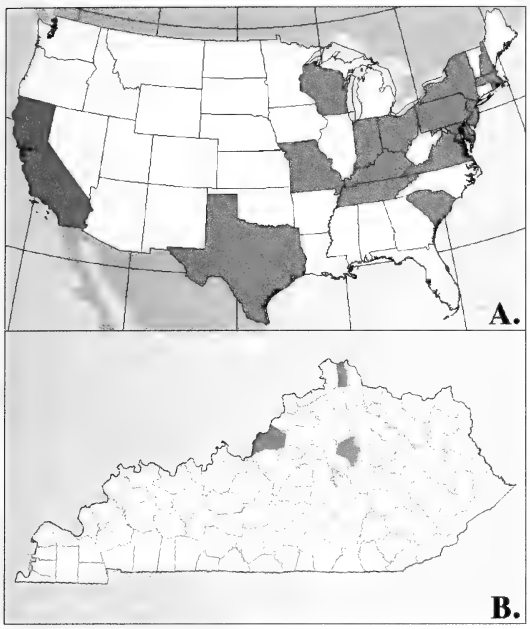

Figure 3. A. The wood snail has successfully colonized most of the northeastern U.S. and has also been found in some western states, including California and Texas (Abbott 1950; Burch 1962; Dundee 1974; Reed 1964). B. In Kentucky, populations of the wood snail have been found in Fayette, Jefferson, and Kenton Counties. (Maps courtesy of the online National Atlas of the United States [2005].)

terials or of British snails in imported ivy (Barber 1918; Howe 1898). After the turn of the century, imported shrubs from the Netherlands and Ireland were probably the source of other snail populations discovered in Virginia and Massachusetts, respectively (Reed 1964). By 1974, populations of wood snails had been documented in at least 15 states and throughout the northeastern U.S. (Figure 3A).

KENTUCKY POPULATIONS

Documentation of land snail diversity and distributions in Kentucky is scanty, but scattered populations of *C. nemoralis* are reported from the state (Figure 3B). Reed (1964) cited a specimen found in Ohio River drift at Louisville, and at least three Lexington collections have been recorded (Branson and Batch 1969; FNMH 2005). Specimens have also been noted from Cincinnati, Ohio (Reed 1964; FMNH 2005), so it is not surprising that a large population of *C. nemoralis* was recently found in northern Kentucky near Ft. Mitchell, Kenton

Figure 4. Living wood snail from Kenton County, Kentucky. These snails have four pairs of tentacles, with the eyes located at the tips of the retractable upper pair. A shorter pair of sensory tentacles bracket the snail's mouth.

County (pers. obs.; see Figure 4). Surveys of snail fauna from Mammoth Cave National Park in Edmonson County and the Doe Run Creek Area of Meade County did not list *C. nemoralis* among the species found, perhaps because these are less disturbed areas than those noted above (Hubricht 1968; Kaplan and Minckley 1960).

ECONOMIC IMPORTANCE

While considered one of the European edible snails, its small size, and the belief that species with striped shells are inferior in flavor have limited the popularity of *C. nemoralis* among gastronomes (Lovell 1884). None the less, the relative hardiness of this species compared to larger species of escargot, the ease of culture, and the nearly worldwide availability has kept them on the lists of species with potential for cultivation (Dees 1970; Thompson 1996).

Though wood snails eat a variety of plant materials, their apparent preference for dead material has limited their impact as agricultural pests (Dees 1970; Thompson 1996). Occasional note has been made of the fact that even in areas with many snails, they appear to do little damage to the flora (Abbott 1950; Brooke 1897; Judd 1953). However, in high enough densities, they have the potential to damage landscaping or crops. For example, one Virginia population ranged from 50–100

snails per square meter, with an estimated total of 2500–5000 individuals (Stine 1989).

Wood snail shells are carried by some shell dealers, but generally tropical landshells and marine shells are more popular with collectors. At least one U.S. population may have been introduced to serve as a shell source (Cowie and Robinson 2001).

Wood snails also make easy to care for, if unusual, pets. However, most land snails are considered potential pest species by the USDA, and there are restrictions even on state-to-state transport of living snails (Dees 1970; Thompson 1996), dashing the hopes of those in the exotic pet industry hoping to spark a nationwide snail craze.

ECOLOGICAL AND GENETIC STUDIES

The variety of shell colors seen among banded wood snails has long fascinated naturalists, and many papers catalogue diversity within populations (Brooke 1897; Howe 1898; Johnson 1928; Judd 1953). The genetics of most color variations have been determined via crossing studies (Cain et al. 1968). At least five shell color loci are linked into a "supergene" (Jones et al. 1977). These control the shell's base color and four banding features: presence or absence, intensity of band and lip color, whether bands are continuous or dotted, and their spread (Jones et al. 1977). Four other unlinked loci also affect banding, with the number of bands controlled by two, one controlling darkening along the length of the bands, and one determining whether bands are black or orange (Cain et al. 1968; Jones et al. 1977). Epistasis between some loci also plays a role (Jones et al. 1977). Considering that there are no fewer than six alleles for base color of the shell, and that banding is affected by at least eight loci and 18 alleles (Jones et al. 1977), it is not surprising that early workers enumerated hundreds of shell varieties (Howe 1898).

Researchers have wondered how such high levels of variation are maintained. With long distance gene flow often limited by the slow spread of individuals, and many populations founded by small numbers of snails, one would expect to commonly see fixation of shell morphs through loss of alleles. However, fixed populations are rare. For example, a survey of 1000 French populations revealed only two that were monomorphic for shell coloration (Murray 1964). In a similar survey of 3000 British populations, fewer than 20 were monomorphic (Jones et al. 1977). Two factors are thought to play a crucial role in maintaining this diversity. Because these snails are hermaphrodites, mating is possible between any two individuals, increasing the potential allele combinations available to offspring (Murray 1964). Also, wood snails generally mate at least twice prior to laying eggs, and can store sperm from multiple matings, effectively increasing the population size (Murray 1964). Thus, even small populations of snails may harbor more genetic diversity than would be seen in other types of organisms.

Founder effects do have an impact on diversity, though, especially in U.S. populations, most of which arose from introductions of small numbers of individuals (Brussard 1975). A study scoring shell polymorphisms and nine isozyme loci showed that the major differences between U.S. populations seemed to be based on which part of Europe the snails had been introduced from, rather than the environment they were currently in (Brussard 1975). Later isozyme studies have also supported the founder effect as having a major impact on the genetic variation within U.S. populations (Selander and Foltz 1981).

Climate also has great influence on the diversity of shell colors. Wood snails, commonly found in cool temperate climates, are sensitive to overheating (Arnold 1969; Jones et al. 1977). One study of populations on sand dunes found a disproportionate number of brown and pink shelled individuals dying from heat shock (Jones et al. 1977). Climatic selection is thought to play a major role in large-scale patterns of shell color, with pale shells being selected for in hotter climates (Jones et al. 1977). Indeed, there is a cline for shell color across Europe, and in the hottest parts of their European range, yellow shelled wood snails are the most common type (Jones et al. 1977). Additionally, observation of shells dug from archaeological sites in England shows that, historically, brown shells were more common during periods with colder climates (Jones et al. 1977). However, interpretation of the interplay between climate and color is complicated by the fact that small scale environmental conditions may also have an effect

(Arnold 1968, 1969; Jones et al. 1977). For example, even in regions with generally warm climates, brown shells may be favored in certain cool, shady microhabitats, because brown individuals absorb heat faster than pale individuals and can thus become active more quickly (Jones et al. 1977).

Early workers observed that wood snails had a tendency to "mimic" their backgrounds, with the shell colors that best blended into the background being the most common (Howe 1898). Later workers have shown that visual selection by predators can produce this effect (Currey et al. 1964; Davison 2002; Jones et al. 1977). In Great Britain, song thrushes are efficient snail predators, crushing the shells on stones to get to the snail. Birds see in color, and in areas where song thrushes are common, shells which contrast with their backgrounds are preferentially eaten (Currey et al. 1964; Jones et al. 1977). Other predators such as mammals and glowworms have also been shown to prefer certain shell morphs over others (Jones et al. 1977).

With such a wealth of information on the genetic control of shell color, diversity of natural populations, and factors influencing shell morphs, wood snails have become wonderful model systems for study of evolutionary mechanisms and ecological genetics (Davison 2002). These organisms have the added advantage of being common and easy to work with in both the field and the lab.

ECOLOGICAL IMPACT

Some authors have expressed concern about the potential impact of non-native snails upon populations of our native species (Cowie and Robinson 2001; Mead 1971). Several years ago, wood snails were introduced to the Stone Lab area of Gibraltar Island (Ohio) via landscaping activities, and Dr. Michael Hoggarth of Otterbein College has since noted an apparent decrease in the numbers of native snails seen there (pers. comm.). This is an issue that calls for further study.

CONCLUSION

While recent surveys of *C. nemoralis* populations in the U.S. are limited, it is obvious that this species has become widely established. Several populations from Kentucky have been noted, and further searching would undoubtedly uncover more. Though the species is apparently not a major agricultural pest, the potential impact of these very successful aliens on our native snail populations should be of concern. However, now that the wood snail has come to stay, its potential for use in the classroom or for ecological genetics studies is an opportunity not to be overlooked.

ACKNOWLEDGMENTS

Thanks to Dr. Michael Hoggarth of Otterbein College in Westerville, Ohio for critiquing this paper; to Merritt Gillilland of Michigan State University for advice on finding some of the early literature on *Cepaea* introductions in the U.S.; to Dr. Debra Pearce, Northern Kentucky University, for aid; and to Dr. John Thieret and the staff of the Lloyd Library in Cincinnati for providing access to a variety of delightful and very vintage malacological works.

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Location of Rediae of *Proterometra macrostoma* (Trematoda: Azygiidae) in the Snail *Elimia semicarinata* (Gastropoda: Pleuroceridae), and Daily Emergence of its Cercaria

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ABSTRACT

The objectives of this study were to describe the (1) location of *Proterometra macrostoma* rediae in the snail intermediate host, *Elimia semicarinata*, and (2) daily emergence of cercariae from individual snails over 3 weeks. Decalcification and histological sections of infected snails revealed rediae restricted to the bottom whorl within the mantle cavity in close association with the host gills. A continuous, low-level emergence of cercariae (0.25–0.833 larvae/snail/day) was observed over 21 days. Both moderately frequent (7–12 days) and infrequent (2–6 days) shedding of cercariae were observed (i.e., mean ± SE = 7.3 ± 0.4 days; range = 2–12 days), but no consistent pattern was apparent. The average ± SE number of rediae was significantly smaller in snails shedding only 2–6 days (11.9 ± 2.9) than snails shedding between 7–12 days (35.2 ± 8.5) at the termination of the experiment.

INTRODUCTION

The original work on morphology and general biology of the cercaria of *Proterometra macrostoma* was completed by Horsfall (1934) and Dickerman (1945). Horsfall (1934) noted the presence of larval forms of *P. macrostoma* in the snail body cavity. Dickerman (1945), based on his study of crushed snails, indicated that the size and number of emerging cercariae may cause the body cavity wall to rupture, thus introducing cercariae into the mantle cavity from where they can exit into the outside environment. However, Hyman (1967) and Voltzow (1994), in extensive reviews of prosobranch anatomy, did not mention the presence of a body cavity associated with these snails. Thus, these gross observations should be reassessed by histological techniques aimed at describing the correct location of the *P. macrostoma* rediae and cercariae within their snail host.

Lewis (1988) determined that an average of only 0.35 and 0.29 *P. macrostoma* cercariae/snail were released daily in the field and laboratory from sample populations of snails, respectively. This low production of *P. macrostoma* cercariae was also documented in a 3-week study of daily emergence (Lewis et al. 1989). According to Lewis et al. (1989), this is 100–1000-fold less than most other digeneans, representing an adaptation for the creation of a few, large, conspicuous cercariae. However, no hypothesis was offered for this low cercarial output. Evaluation of cercarial emergence patterns from individual snails and a reexamination of *P. macrostoma* redial stages may further our understanding of this phenomenon.

The objectives of this study were (1) to determine the location of *P. macrostoma* rediae in the snail intermediate host and (2) to describe the daily emergence of cercariae during 3 weeks from individual snails naturally infected with this worm.

MATERIALS AND METHODS

Snails of the species *Elimia semicarinata* were collected from North Elkhorn Creek in Scott County, Kentucky (lat 38°11′00″ N, long 84°29′19″ W), during summer 2004. They were then screened for patent infections (i.e., shedding cercariae) as described by Rosen et al. (2000). Thirty-six infected snails were simultaneously fixed and decalcified in Cal-Ex II (Fisher) for routine paraffin sectioning to describe redial location in the snail host. Serial sections (5–10 μ) were stained with hematoxylin and Gomori's trichrome. Emergence of cercariae was assessed for 36 additional infected snails that were individually isolated, held at 20°C under a 12 hr light : 12 hr dark cycle, and checked at the end of each of these

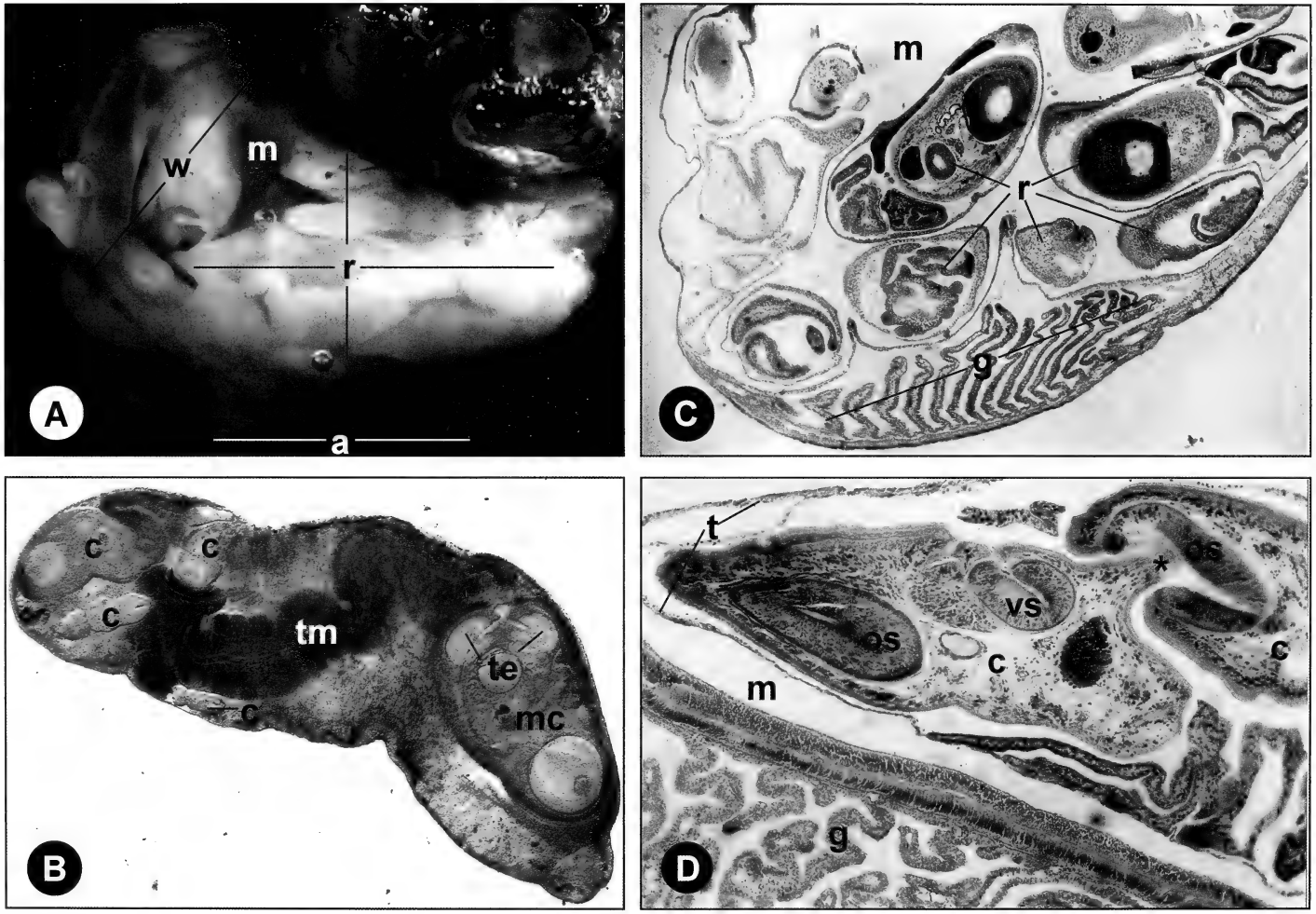

Figure 1. (A) Decalcified *Elimia semicarinata* showing *Proterometra macrostoma* rediae in bottom whorl; 23×. (B) Developing cercariae in a *P. macrostoma* redia; 40×. (C) Histological section of bottom whorl of *Elimia semicarinata*; 40×; 10 μ; hematoxylin and Gomori's trichrome. (D) Cercarial interaction within a *P. macrostoma* redia; 100×; 10 μ; hematoxylin and Gomori's trichrome. Abbreviations: a = snail aperature; c = cercaria; g = gills; m = mantle cavity; mc = mature cercaria; os = oral sucker; r = rediae; t = tegument of redia; te = testes; tm = tail of mature cercaria; vs = ventral sucker; w = bottom whorl of snail; * = oral sucker of one cercaria adhering to body of a second cercaria.

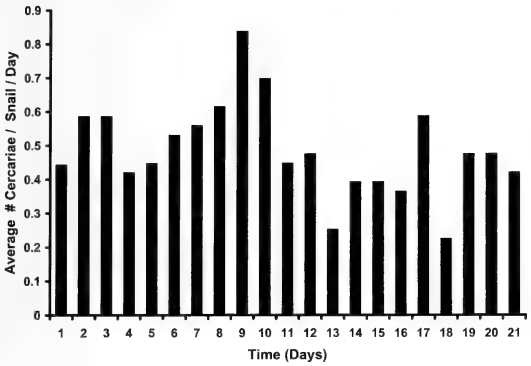

Figure 2. Average number of *Proterometra macrostoma* cercariae/snail shed daily over 21 days for 36 snails.

12 hr periods for 21 days. Snails were transferred into containers with new dechlorinated water at the end of each 12 hr period. At the termination of the experiment, snails were dissected to assess the number of rediae in each.

RESULTS

Decalcification of the snail shell revealed rediae in the bottom whorl of the host (Figure 1a). Rediae usually contained only 5–6 cercariae at markedly different stages of development (Figure 1b). Histological sections of the whorl region revealed numerous rediae within the mantle cavity in close association with the gills (Figure 1c). Cercariae in rediae were occasionally observed adhering to one another with their oral suckers (Figure 1d).

The majority of cercariae (365/369; 98.9%) were released during the 12 hr dark cycle of each 24 hr period. An oscillating pattern of continuous, low-level cercarial release from our pooled snail population was demonstrated over 3 weeks, with averages ranging between 0.250 and 0.833 cercariae/snail/day (Figure 2). Both infrequent (2–6 days; Figures 3a and 3b) and moderately frequent (7–12 days; Figures 3c and 3d) cercarial shedding were noted from individual snails over 21 days, and no consistent pattern was apparent. Individual snails shed cercariae on average ± SE = 7.3 ± 0.4 days of the 21-day experiment (range 2–12 days; Figure 4). Snails shedding cercariae only 2–6 days (e.g., Figures 3a and 3b) of the 21 day experiment contained an average ± SE of 11.9 ± 2.9 rediae, while snails shedding between 7–12 days (e.g., Figures 3c and 3d) possessed 35.2 ± 8.5 rediae. These values were significantly different when assessed with a Student's *t*-test ($t = 2.218$; df = 32; $P = 0.034$).

DISCUSSION

Our histological observations established that *P. macrostoma* rediae and their developing cercariae inhabit the mantle cavity of *E. semicarinata*. Once cercariae are released into this space by rediae, the route for emergence is clear as the mantle cavity communicates directly with the outside environment. One possible consequence associated with this host locale is that *P. macrostoma* cercariae might not encounter sudden osmotic stress when released from the snail host as previously reported (Braham et al. 1996). The ciliated gills in prosobranchs like *E. semicarinata* establish a unidirectional circulation of freshwater through this cavity for respiration (Voltzow 1994). This circulation would likely expose *P. macrostoma* cercariae within rediae to a hypotonic environment prior to emergence, but samples of fluid from this space will be required for confirmation of its hypotonic nature. How such an environment might impact cercarial development in rediae remains unknown.

Theron (1981), in a long-term study of snails experimentally infected with *Schistosoma mansoni*, linked peaks and valleys in the shedding of cercariae over 3 months to a succession of cercarial generations, the valleys being intervals of lower production. No such peaks and valleys were obvious in our 3-week study of cercarial shedding from snails infected with *P. macrostoma*. By contrast, a continuous and low-level release of *P. macrostoma* cercariae from our sample population of snails over 21 days was observed and was similar to that previously reported by Lewis et al. (1989). Unlike Theron's (1981) infections, our snails were not synchronized as to time of initial infection.

One snail shed cercariae 12/21 days in this study, while the remaining snails shed only between 2 and 11/21 days. This rather infrequent daily emergence was likely associated with the markedly small number of large cercariae produced within rediae of *P. macrostoma*. Cercariae within a redia were at noticeably different stages of development as previously reported by Horsfall (1934), and sev-

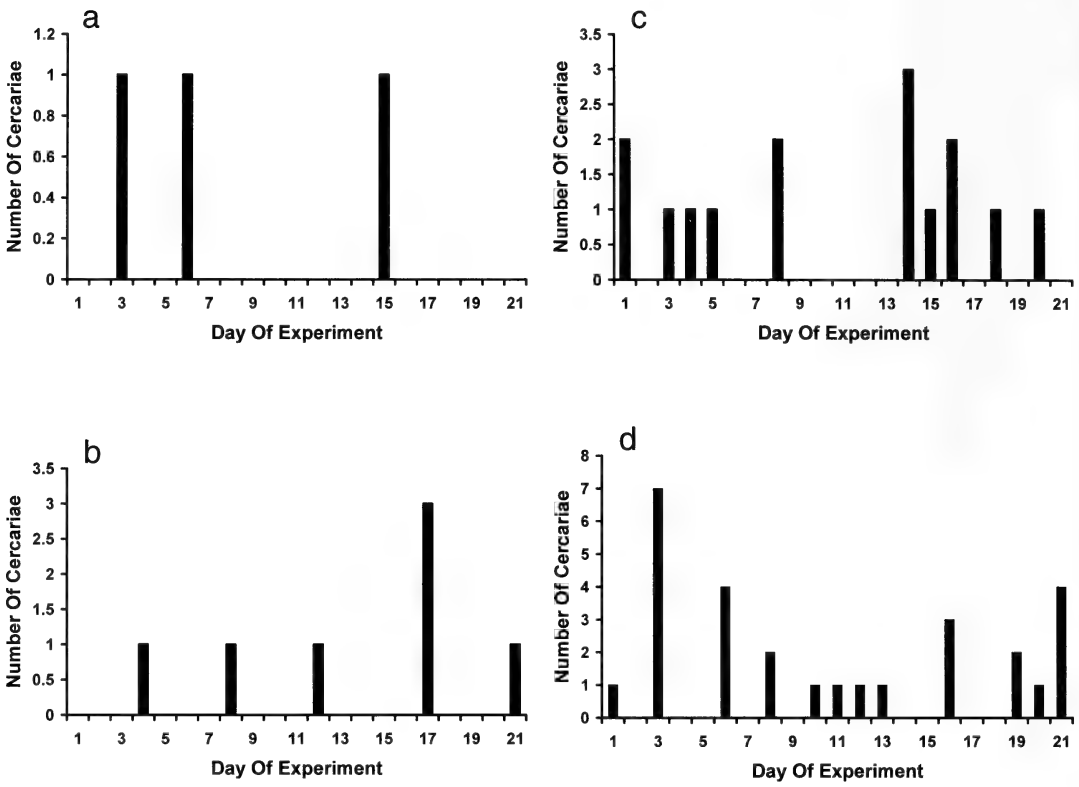

Figure 3. Examples (4/36) of number of *Proterometra macrostoma* cercariae shed by individual snails over 21 days. (A) Snail 4 shed 3 cercariae from 11 rediae. (B) Snail 14 shed 7 cercariae from 4 rediae. (C) Snail 13 shed 15 cercariae from 28 rediae. (D) Snail 11 shed 28 cercariae from 26 rediae.

eral days are probably required for the next stage to mature following the release of the most advanced (i.e., with functional genital organs) cercaria. Even snails with many rediae conformed to this limited pattern of cercarial emergence. This slow development may be linked to a variety of factors. Mature rediae of *P. macrostoma* lack a gut unlike rediae of most digeneans, and thus acquisition of nutrients is dependent solely on mediated transport by the tegument (Uglem 1980). The mantle cavity habitat confirmed in this study, by contrast to preferred redial sites, such as the snail gonad and digestive gland (Fried 1997), may contain fewer nutrients for asexual reproduction of cercariae in such rediae, but this will require future verification.

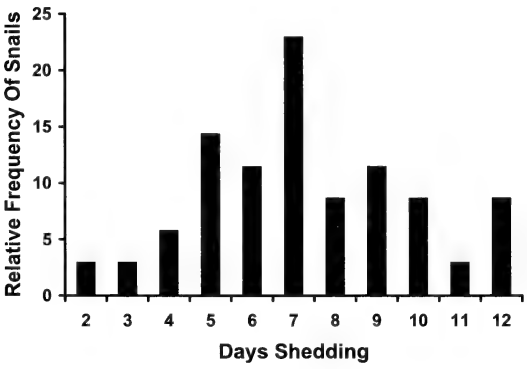

Figure 4. Relative frequency of cercarial shedding from 36 snails infected with *Proterometra macrostoma* over 21 days.

ACKNOWLEDGMENTS

This study was supported by a grant from the Undergraduate Research and Creative Projects Program (URCPP) at Berea College to Ronald Rosen. We acknowledge the late Gary. L. Uglem, who provided the first author much insight and support with the *P. macrostoma* system; and Dr. Bernard Fried, Department of Biology, Lafayette College, and Dr.

Allen Shostak, Department of Biological Sciences, University of Alberta, for reviewing this paper.

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J. Ky. Acad. Sci. 66(2):94–100. 2005.

Effect of Light Wavelength and Osmolality on the Swimming of Cercariae of *Proterometra macrostoma* (Digenea: Azygiidae)

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ABSTRACT

The objectives of this study were to describe the effect of light wavelength and osmolality on the vertical swimming distance of the cercaria of *Proterometra macrostoma.* Significant differences were found in the average swimming distances and electrical burst activity in the tail of cercariae exposed to red vs. blue, green or white light, but no difference was observed when the last three colors were compared to one another with one exception. Cercarial swimming decreased slightly after 12 hr PE (post-exposure) in artificial pond water (15 mOsm) and artificial snail water (102 mOsm). By contrast, a significant reduction in swimming distance was observed in distilled water (0 mOsm) and artificial snail water with two different concentrations of mannitol (180 and 267 mOsm).

INTRODUCTION

Production of the furcocystocercous cercaria of *Proterometra macrostoma* is 100–1000 fold less than most other digeneans (Lewis et al. 1989). However, this cercaria possesses several adaptations that increase the probability that it will remain infective and be ingested by centrarchid fish definitive hosts. The retraction of the cercarial body into its tail prior to emergence from the snail host serves to protect the body from osmotic stress subsequently encountered in a freshwater environment (Braham et al. 1996). In addition, the conspicuous swimming behavior and size (4–5 mm) of this cercaria serve to attract appropriate fish definitive hosts.

Infectivity of *P. macrostoma* cercariae is retained over 14 hr in hypotonic artificial pond water as long as the cercarial body remains in the tail chamber (Braham et al. 1996). The primary source of this protection has been linked to a continuous barrier provided by the basal membrane associated with the tegument of the cercarial tail (Braham and Uglem 2000). Such protection is likely essential for the perpetuation of *P. macrostoma,* as snails infected with this species release only one or two cercariae at a time often separated by several days without releasing cercariae. The basal membrane may be able to withstand even more extreme hypotonic and hypertonic osmolalities than naturally encountered, given the limited reproductive capacity of this species. Such protection may be evaluated indirectly within the initial 12 hr PEm (post-emergence) of the cercaria from its snail intermediate host by placing cercariae in a broad range of osmolalities and observing temporal changes in cercarial swimming burst length and infectivity. The latter may be assessed by the activation and emergence of the cercarial body from its tail when exposed to low pH, which simulates conditions found in the stomach of the fish definitive host (Horsfall 1934; Rosen et al. 2000).

According to Prior and Uglem (1979), the tail of this cercaria is an autonomous locomotor organ, and the vertical swimming phase is initiated through tactile stimulation of the tail. Following such stimulation, the regular, alternating pattern of the swim-sink cycle in *P. macrostoma* cercariae has been linked to a possible endogenous oscillator system located in the transverse band of the tail. Electrical potentials are generated from this region and spread to the anterior end of the tail. This electrical activity can be modulated by light intensity. Lewis (1988) found an inverse relationship between light intensity and electrical burst activity in the tail of *P. macrostoma.* Increases in light intensity resulted in shortened electrical burst durations (BD) and longer interburst intervals (IBI), while the opposite was true for sudden decreases in intensity. However, no effort was made to evaluate individual components of the visible spectrum (con-

trolling for light intensity) on this swimming behavior and associated electrical burst activity in the cercarial tail.

The objectives of our study were to determine (1) the range of osmolalities tolerated by the cercarial tail as demonstrated through swimming longevity and the subsequent emergence of the cercarial body from its tail and (2) if a graded swimming response and associated electrical activity in the cercarial tail result from exposure of cercariae to selected wavelengths of the visible spectrum.

METHODS

Snails, *Elimia semicarinata,* were collected from North Elkhorn Creek in Scott County, KY (38° 11′ 00″, 84° 29′ 19″ W), during summers 2000 and 2004. They were then screened for patent infections (i.e., shedding cercariae) as described by Rosen et al. (2000). To assess the effect of osmolality on cercarial swimming, freshly released cercariae (i.e., less than 1 hr PEm) were placed into beakers containing each of the following hypotonic or hypertonic solutions; distilled water (0 mOsm; hypotonic), artificial pond water (APW; 15 mOsm; 0.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2$, and 0.025 mM $MgCl_2$; hypotonic), artificial snail water (ASW; 102 mOsm; 15.0 mM NaCl, 1.0 mM KCl, 5.0 mM $CaCl_2$, 0.5 mM $MgCl_2$; hypotonic), and ASW with 100 and 200 mM mannitol, resulting in 180 and 267 mOsm hypertonic solutions, respectively. A crude extract of snail fluid was made by removing the snail shell in ASW and blotting the body for 5 sec on filter paper. Next, snail bodies were placed in a tissue grinder, the resulting fluid centrifuged with an Eppendorf microcentrifuge for 1 min, and the supernatant osmolality determined. This value served as the isotonic osmolality baseline for the experiment. Final osmolalities of the test solutions, North Elkhorn Creek water, and the snail fluid were determined using a Wescor Vapro vapor pressure osmometer. Individual cercariae were placed into separate 2-liter graduated cylinders filled with the test solutions to assess swimming. Vertical swimming distances were obtained by dividing the distance traveled by a cercaria (according to the horizontal ml marks on the cylinder) by five based on the actual distances between these graded marks (e.g., 1.0 ml = 0.2 mm). These distances were measured in five replicate experiments on an hourly basis for 12 hr. A one-way ANOVA was used to compare mean swimming distances at 6 and 12 hr PE (post-exposure). The following technique was used to assess the effect of long-term exposure of cercariae to different osmolalities on the subsequent emergence/infectivity of the cercarial body from its tail. Additional cercariae (6–8/osmolality) from each of the test solutions were placed in 10 ml beakers filled with a solution adjusted to pH 2.0 at 6 and 12 hr PE. for 1 hr to simulate conditions found in the host fish stomach following ingestion of cercariae. The numbers of completely emerged cercarial bodies were recorded at the termination of the 1-hr incubation.

A preliminary experiment demonstrated that there was no decrease in cercarial swimming during the initial 9 hr PEm at 15°, 20°, and 25°C. Thus, cercariae, less than 6 hr PEm, were individually isolated in 2-liter graduated cylinders filled with aerated and filtered North Elkhorn water to assess the effect of light wavelength on vertical swimming distance. Broad spectrum, 25-watt fluorescent lights double-wrapped in 2-mm red, green, or blue filters (Edmond Scientific F35235-F35137) or black fiberglass window screen were placed horizontally on stands above the cylinders and adjusted to achieve a light intensity of 11.5 foot candles (fc; Extech Light Meter) at the 2-liter mark The light intensity selected approximated daylight field values (14.5 fc) obtained at North Elkhorn Creek at 0.6 m depth. Twenty-three cercariae were exposed in 7-min intervals to a sequence of red-blue-red-white-red-green-red light conditions. Each 7-min time span consisted of a 2-min acclimation period followed by a 5-min recording period. A single swimming burst distance was recorded at the beginning of each minute of the 5-min recording interval for each light treatment, and an average distance, using the aforementioned conversion factor, was calculated from these five observations.

Cercarial electrical activity was subsequently recorded under the same light condition sequence and timing using the technique of Uglem and Prior (1983). Electrical recordings were made by placing cercariae in fingerbowls with APW. The large size of the *P. macrostoma* cercaria made it possible to pin the up-

per body and tail of individual worms to wax in a petri dish, and apply a suction electrode to the transverse band of the cercaria at the junction of the body and the bifurcated tail. White fiber-optic 8.0-mm light cables were covered with the aforementioned filters and adjusted to deliver an intensity of 11.5 fc for each color. An AC amplifier at 10 Hz was used to amplify signals. The average (10 replicates) duration of electrical burst activity under each light condition was recorded. A one-way, repeated-measures ANOVA was used to compare (1) the mean swimming burst distance and electrical burst activity in the presence of the four red-light treatments and (2) the mean of the four red-light treatments to the individual means of the blue, white, and green regimens. Paired Student's *t*-tests were used to determine possible differences between each pair of conditions in the latter analysis.

RESULTS

The osmolalities of North Elkhorn water and snail extract were determined to be 10–15 and 157 mOsm, respectively. The former closely approximated APW (15 mOsm), while the latter was between ASW (102 mOsm) and ASW supplemented with the 100 mM mannitol (180 mOsm). Observation of cercariae from the test solutions with a dissecting microscope at 12 hr PE revealed significant swelling and shrinkage of the tail in the 0 and 267 mOsm solutions, respectively. Swimming burst distances of cercariae in the five tested osmolalities over 12 hr PE are summarized in Figure 1. One-way ANOVA analysis revealed significant differences between the mean swimming distances at both 6 ($F = 10.064$; $df = 4, 20$; $P < 0.001$) and 12 hr PE ($F = 6.622$; $df = 4, 20$; $P < 0.001$). Swimming of cercariae in APW and ASW remained relatively unaffected, with a small decrease at the termination of the experiment (Figure 1). Cercariae in 267 mOsm ceased swimming after only 4 hr, while markedly reduced swimming was obvious in distilled water and 180 mOsm (Figure 1). All cercarial bodies placed in the pH 2.0 solution successfully emerged from their tails at 6 hr PE, while 97% emerged after 12 hr PE. The lone exception occurred in the 267 mOsm solution, where six out of seven bodies emerged.

Most cercariae remained motionless on the bottom of the cylinder when exposed to white, blue, or green light at the beginning of the 2-min acclimation (i.e., non-recording) period of our light experiment. Conversely, cercariae swam several extended bursts during this acclimation period when first exposed to red light. No significant differences were observed in the mean swimming distances ($F = 0.107$; $df = 3, 66$; $P = 0.956$; Figure 2a) or electrical burst activity ($F = 1.172$; $df = ; 3, 27$; $P = 0.339$; Figure 2b) between the four red-light treatments. Mean swimming distances ($F = 28.176$; $df = 3, 66$; $P < 0.001$; Figure 2a) and electrical burst activity ($F = 9.664$; $df = 3, 27$; $P < 0.001$; Figure 2b) were significantly greater under the red-light treatment compared to the blue, white, and green conditions. Swimming distances and electrical burst activity were not significantly different when the blue, white, and green conditions were compared to each other, the lone exception being the blue vs. white treatment in the electrical activity analysis (Table 1); all three were significantly different when individually compared to red light (Table 1).

DISCUSSION

In our experiments, osmolalities lower than 15 mOsm and higher than 102 mOsm significantly affected the vertical swimming distance of the cercaria of *P. macrostoma.* These results suggested that the continuity of the protective basal membrane identified in the study of Braham and Uglem (2000) became compromised in the more extreme hypotonic and hypertonic solutions. This was visually corroborated by the swelling and shrinkage of cercarial tails from the 0 and 267 mOsm solutions at 12 hr PE, respectively. Thus, the plasticity of this tail barrier seems limited to the range of osmolalities (e.g., North Elkhorn Creek water and snail fluid; 15–157 mOsm) these cercariae encounter in nature. None of the tested osmolalities inhibited the activation and subsequent emergence of the *P. macrostoma* cercarial body from its tail. However, as the conspicuous swimming of the tail serves to attract appropriate centrarchid fish hosts, its loss of function negates the continued viability/infectivity of the cercarial body.

Our results indicated that cercarial swimming and electrical activity in the tail of the

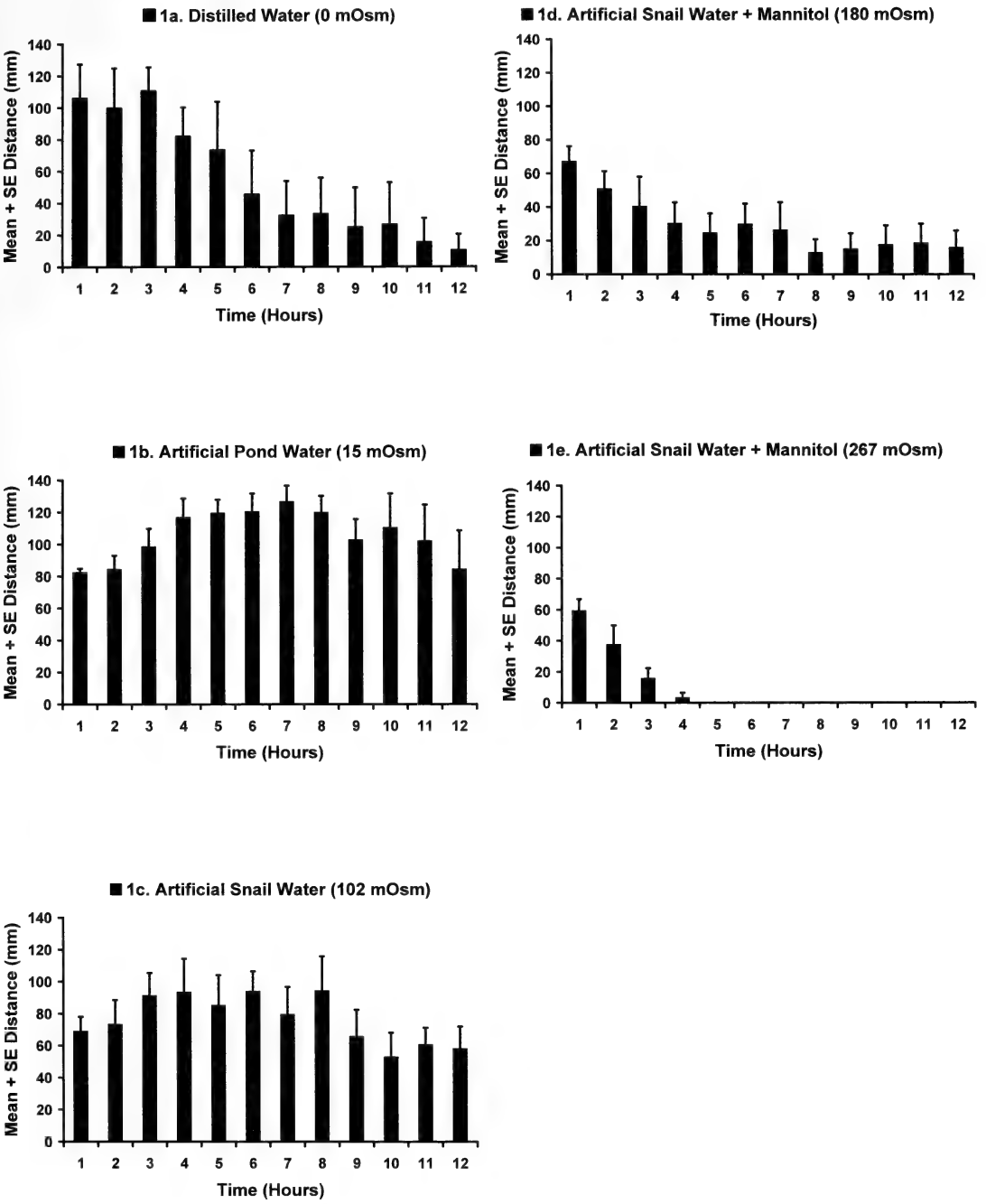
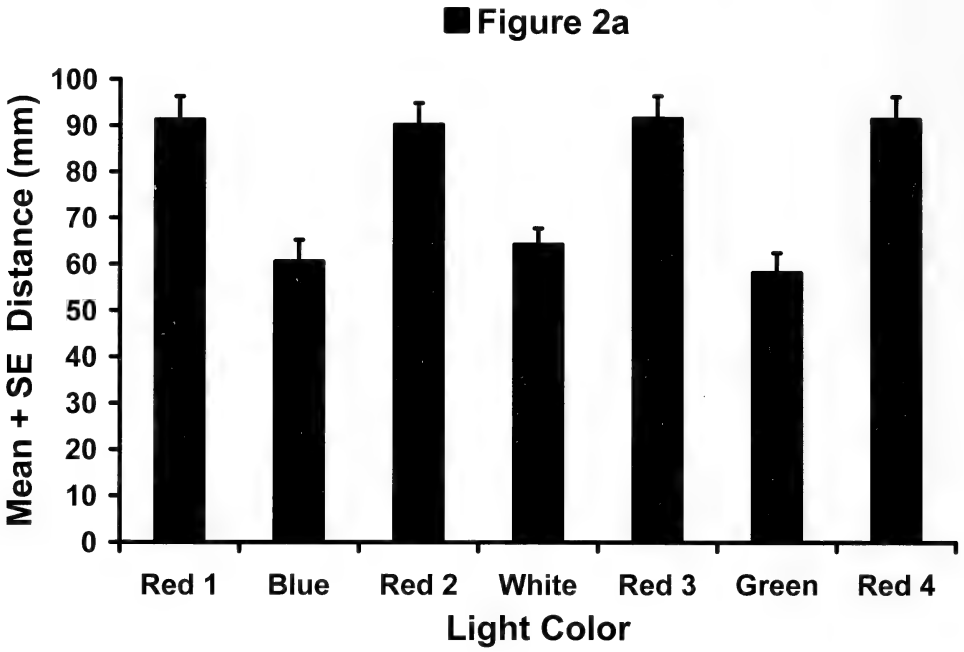


Figure 1. Mean + SE vertical swimming distances (mm) for *Proterometra macrostoma* cercariae over 12 hr in: (1a) Distilled Water; 0 mOsm; (1b) Artificial Pond Water; 15 mOsm; (1c) Artificial Snail Water; 102 mOsm; (1d) Artificial Snail Water + Mannitol; 180 mOsm; (1e) Artificial Snail Water + Mannitol; 267 mOsm.

P. macrostoma cercaria can be modulated by exposure to red light or collectively the shorter wavelengths of the visible spectrum while controlling for light intensity. Light wavelengths are absorbed differentially with increasing water depth, and longer light wavelengths like red are absorbed much more rapidly in water than shorter wavelengths



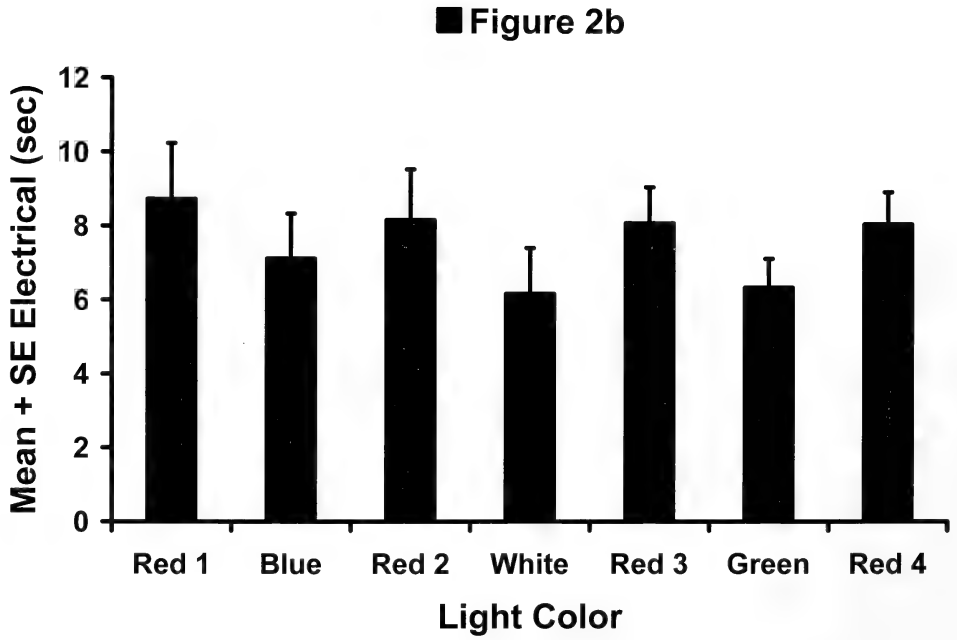


Figure 2. Mean + SE swimming distance (2a) and electrical burst duration (2b) for *Proterometra macrostoma* cercariae under red, blue, white, and green light conditions.

Table 1. Paired Student t-test comparisons of mean swimming distances and electrical burst activity of *Proterometra macrostoma* cercariae under the four tested light conditions.

Comparisons	Vertical swimming distances		Electrical burst activity	
	t (df)	P	t (df)	P
Red vs. Blue	6.866 (22)	< 0.001*	3.289 (9)	0.009*
Red vs. White	7.601 (22)	< 0.001*	6.232 (9)	< 0.001*
Red vs. Green	6.603 (22)	< 0.001*	4.436 (9)	0.002*
Blue vs. White	1.427 (22)	0.168	2.613 (9)	0.028*
Blue vs. Green	0.514 (22)	0.613	1.456 (9)	0.179
White vs. Green	1.527 (22)	0.141	0.140 (9)	0.0891

* Significantly different at $P \leq 0.05$

(Cole 1983) and would thus be less likely to reach and activate a possible cercarial light receptor associated with *P. macrostoma* swimming. Exposure of cercariae to this red light produced significant increases in vertical swimming distances and associated electrical burst duration in the tail of *P. macrostoma* cercariae when compared to the other tested (and shorter) light wavelengths which penetrate deeper into the water column. With regard to these shorter wavelengths, a graded swimming response to this range of the visible spectrum was unsupported due to the absence of significant differences among the blue, white, or green light regimens. This suggested that any shorter light wavelengths reaching the *P. macrostoma* cercariae will produce similar decreases in swimming burst duration. Intense acetylcholinesterase activity has been localized in the transverse band of the tail, and acetylcholine has been shown to abolish or reduce electrical burst activity in the tail of *P. macrostoma* (Uglem and Prior 1983). It is possible that the release of this neurotransmitter was triggered by exposure of cercariae to blue, white, or green light as evidenced by the reduced activity of cercariae exposed to these light regimens.

The maximum swimming burst established for this cercaria when exposed only to red light likely simulates nocturnal conditions due to the diminished penetration of this wavelength in the water column and coincides with the nocturnal shedding of *P. macrostoma* in nature (Lewis 1988). This extended swimming would assist in attracting night-feeding hosts such as warmouth, *Lepomis gulosus* (Larimore 1957). However, this cercaria can continue swimming up to 14–20 hr (Braham et al. 1996), well into daylight hours in the field during spring and summer months. In this regard, it has been found that *Schistosoma haematobium* (Haas et al. 1994) and *Cryptocotyle lingua* (Chapman 1974; Rea and Irwin 1992) exhibit an increased swimming response to brief, dark stimuli known as the "shadow response." Rea and Irwin (1992) found that exposure to shadowed light increased the attachment of 12–13 hr *C. lingua* cercariae to their fish host. Fish casting a shadow during daylight hours on *P. macrostoma* would trigger greater vertical swimming. Such a response would make *P. macrostoma* cercariae more obvious for subsequent ingestion by day-feeding hosts such as longear sunfish, *Lepomis megalotis* (Becker 1983).

ACKNOWLEDGEMENTS

This study was supported by a grant to Ronald Rosen from the Undergraduate Research and Creative Projects Program (URCPP) at Berea College. We acknowledge the late Gary L. Uglem, who provided the first author much insight and support with the *P. macrostoma* system; Dr. Gene Chao, Department of Psychology, Berea College, for assisting us with our electrophysiology techniques; Finally, Dr. Bernard Fried, Department of Biology, Lafayette College, Easton, Pennsylvania, and Dr. Allen Shostak, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, for reviewing this paper.

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Natural and Experimental Infections of Centrarchid Fishes by the Digenetic Trematode *Proterometra macrostoma*: Detection of New Infections and Host Histopathology

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ABSTRACT

The objectives of this study were (1) to use egg development in the adult worm of *Proterometra macrostoma* as a means to delineate new infections of centrarchid fishes with this digenean and (2) to assess histopathology associated with infection of the fish host. An equal mix of adult worm ages/types based on eggs stages was produced by exposing individual hatchery-reared bluegill to the same number of cercariae at weekly intervals. These results confirmed the use of egg stages for differentiating recent vs. older infections in fishes from North Elkhorn Creek, Scott County, Kentucky, during June and July 2002. Between 50.5% and 74.4% of worms recovered from naturally infected warmouth, bluegill, and longear sunfish lacked eggs or possessed eggs in early cleavage with a large vitelline mass implicating summer as a period for new infections of fishes with this worm. A proportionately smaller number of *P. macrostoma* containing eggs in late cleavage (11.4–20.5%) or with miracidia (14.6–29.7%) was also found at this time. In experimental infections of hatchery-reared bluegill, damage to the host mucosa appeared restricted to the attachment site. The oral sucker of adult worms constricted the mucosa of the host esophagus and stomach, causing hemorrhaging and epithelial necrosis. Complete detachment of host tissue in the oral sucker of many worms was confirmed through serial sections, yet no experimentally infected fish died before they were sacrificed at the end of this study.

INTRODUCTION

Proterometra macrostoma (Family Azygiidae) is a digenetic trematode widely distributed in streams and rivers east of the Mississippi River (Riley 1992). The life cycle is indirect, incorporating a snail intermediate host and a centrarchid fish definitive host. The latter becomes infected upon ingestion of the parasite's unusually large cercaria, which swims actively (Prior and Uglem 1979; Uglem and Prior 1983) in the water column. The adult worm then exits from its cercarial tail (Rosen et al. 2000) and attaches to the esophagus and stomach of the fish host.

New infections of definitive hosts by helminths are usually determined by identification of immature individuals. However, the body of the *P. macrostoma* cercaria is progenetic, containing fully developed reproductive structures and often eggs in early cleavage (Riley 1992). The cercarial body also has the same general appearance and size of the adult worm (Horsfall 1934). Thus, different criteria are required for differentiating new vs. older infections of fishes by this species. Rosen et al. (2000) used the stage of egg maturation adapted from Horsfall (1934) as a standard for establishing a time line for adult worm development in experimental infections of hybrid bluegill with *P. macrosotma* cercariae. It is proposed that egg development in *P. macrostoma* can also be applied to delineate periods of new infections of fishes with this worm.

Few helminths infecting the alimentary canal of fishes are pathogenic (Needham and Wooten 1978; Paperna 1995; Williams and Jones 1994). Nothing is known regarding subsequent host pathology following ingestion of the *P. macrostoma* cercaria by centrarchid fish. In a related fish digenean, Sillman (1962) observed that *Azygia longa* tightly adheres to the stomach mucosa with its sucker, causing bruising that enables it to feed on blood. The attachment of this worm induces the formation of raised, circular lesions on the surface of the stomach mucosa (Sillman 1962). Our observations of large numbers (e.g., 200 worms in some hosts) of *P. macrostoma* concentrated at the junction of the esophagus and stomach in naturally infected centrarchid fishes, coupled with small, visible lesions at this

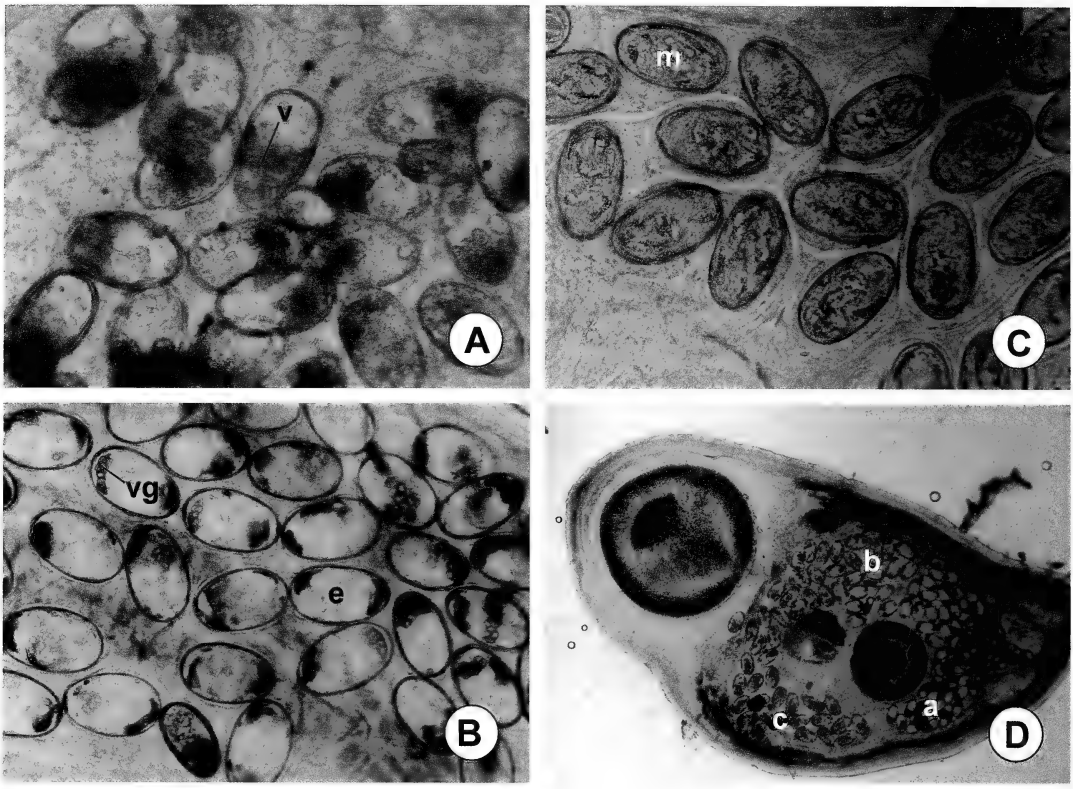

Figure 1. *Proterometra macrostoma* egg developmental stages in adult worms: (A) Stage I, 400× (B) Stage II, 200× (C) Stage III, 200×, and (D) Type III adult, 40×; a = Stage I eggs, b = Stage II eggs, c = Stage III eggs, e = embryo, m = miracidium, v = vitelline material, vg = vitelline granules.

site, suggested the presence of similar pathology.

The objectives of this study were (1) to use egg development in adult worms to assess time of *P. macrostoma* infection in experimentally and naturally infected centrarchid fishes and (2) to describe the histopathology associated with infected fishes.

MATERIALS AND METHODS

Warmouth (*Lepomis gulosus*; N = 18), bluegill (*Lepomis macrochirus*; N = 36), longear sunfish (*Lepomis megalotis*; N = 45), and infected snails (*Elimia semicarinata*; N = 50) were collected by line (fishes) and hand (snails) during June and July 2002 from North Elkhorn Creek (lat 38° 11′ 00″ N, long 84° 29′ 19″ W) in Scott County, Kentucky. Fishes were dissected within 4 hours of their collection, and the mean intensity of infection (i.e., average number of worms per infected fish expressed as mean ± SE) and prevalence (i.e., percentage of infected hosts) determined for each host species. Wet mounts of all living *P. macrostoma* adults recovered from the esophagus and stomach were examined with a compound microscope, and each worm classified as a Type I, Type II, or Type III adult based on the following criteria. Egg stages in adult worms were adopted from Horsfall (1934): (1) Stage I—egg containing a large clear cell at the opercular end and a mass of dark vitelline material at the opposite end (Figure 1a), (2) Stage II—vitelline mass restricted to a few granules around the periphery of the dividing embryo (Figure 1b), and (3) Stage III—shell dark yellow and containing a fully-developed miracidium (Figure 1c). Based on experimental infections of bluegill with *P. macrosotma* at 24.6°C (Rosen et al. 2000), worms lacking eggs or containing only Stage I eggs were classified as Type I (less than 9 days old), worms with Stage I and II eggs as Type II (between 10 and 17 days old), and worms

with all three egg stages as Type III (18 days or older; Figure 1d).

Individual snails were separated into plastic boxes divided into compartments containing filtered stream water, held in an environmental chamber at 20°C with a 12-hr light:12-hr dark cycle, and fed lettuce ad libitum. When cercariae were required for experiments, any previously emerged larvae were removed and the host snails held for 12 to 24 hr under continuous light. The light source was switched off, promoting a copious release of new cercariae within 2 hr for experimental infections of young, hatchery-reared bluegill (Farley and Farley Farms, Cash, Arkansas). The following experiments were designed to establish an equal mix of different age/type worms in individual fish to confirm the use of egg stages for differentiating between new vs. older infections.

In the first experiment, each of four bluegill was individually placed in separate 1-gallon tanks with three cercariae in each tank so parasite ingestion by each host could be visually confirmed. Infections with three additional cercariae were repeated with the same fish 7 days later (i.e., each fish was exposed to a total of six cercariae resulting in 8- and 15-day old worms at the termination of the 15-day experiment). The same protocol was followed in a second experiment with 14 bluegill, but additional infections were conducted at 7 and 14 days following initial exposure (i.e., each fish was exposed to a total of nine cercariae resulting in 6-, 13-, and 20-day old worms at the end of the 20-day study). Following ingestion, fish were maintained within their respective groups in aerated 38-liter aquaria at 24°C and fed Tetramin (Tetra Sales, Blacksburg, VA). At 15- and 20-days following exposure to the initial set of infections, all fish were necropsied and their worm types determined. A Student's *t*-test (15-day experiment) and a one-way ANOVA (20-day experiment) were used to assess possible differences in the mean number of worm types recovered. In addition, the distribution of egg developmental stages (I to III) in each worm type was determined in the 20-day study of bluegill.

Twenty-one additional hatchery-reared bluegill were experimentally infected as previously described with four cercariae each for

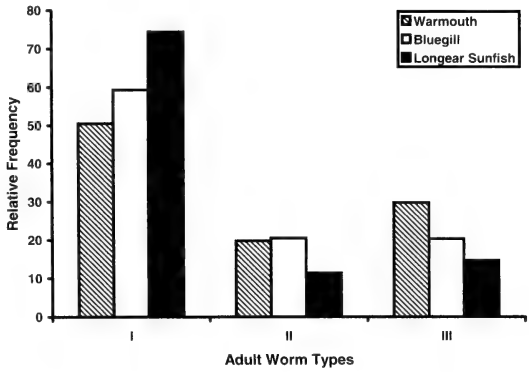

Figure 2. Relative frequency of Type I to III *Proterometra macrostoma* adults from natural infections of warmouth (N = 18 fish and 1524 worms), bluegill (N = 32 fish and 503 worms), and longear sunfish (N = 30 fish and 220 worms).

assessment of histopathology. Seven fish were dissected on days 7, 14, or 24 post-infection (PI), respectively. The esophagus and stomach of each fish were removed, fixed in buffered formalin, routinely processed for paraffin embedding, and serially sectioned with a rotary microtome. Slides were stained with hematoxylin-eosin or hematoxylin in combination with Gomori's trichrome. Several esophagi and stomachs from naturally infected bluegill and warmouth were fixed and assessed in a similar manner.

RESULTS

The mean intensity and prevalence of *P. macrostoma* in naturally infected warmouth, bluegill, and longear sunfish from North Elkhorn Creek were 85.8 ± 16.2 (prevalence = 100.0%), 15.7 ± 2.4 (prevalence = 88.9%), and 7.3 ± 3.1 (prevalence = 67.0%), respectively. Between 50.5 and 74.4% of the *P. macrostoma* populations in these fish were dominated by Type I worms (Figure 2). Smaller numbers of Type II (11.4 to 20.5%) and III (14.6 to 29.7%) worms were also present (Figure 2).

In our experimental infections of bluegill, 95.8% (23/24; 15-day experiment) and 87.3% (110/126; 20-day experiment) of the cercariae originally ingested were recovered as adult worms. The results of a Student's *t*-test showed no significant difference (t = 0.522; 6 df; sig. = 0.620) in the average number of Type I (N = 12) and Type II (N = 11) worms

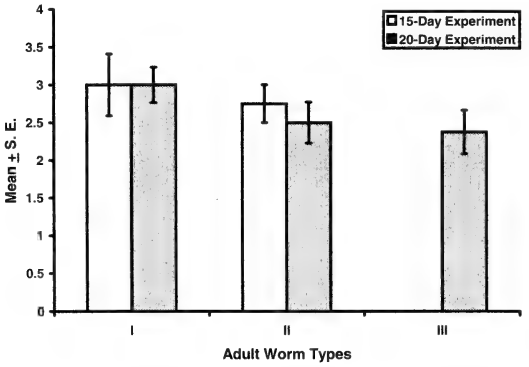

Figure 3. Mean ± SE of Type I and Type II (N = 23 worms; 15-day experiment) and Type 1—III (N = 110 worms; 20-day experiment) *Proterometra macrostoma* adults from experimental infections of bluegill. An individual fish was exposed to three cercariae at one or two weekly intervals resulting in 8- and 15-day infections or 6-, 13-, and 20-day infections, respectively.

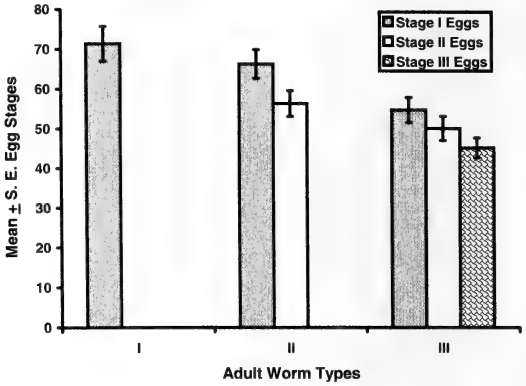

Figure 4. Mean ± SE of egg stages found in Type I to III *Proterometra macrostoma* adults recovered from experimental infections of 14 bluegill.

recovered in our 15-day experiment (Figure 3). Similarly, a one-way ANOVA showed no significant difference (F = 1.607; df = 2, 39; sig. = 0.213) in the mean number of Type I (N = 42), II (N = 35), or III (N = 33) adults recovered in our 20-day experiment (Figure 3). The 8- and 15-day old worms in the 15-day experiment were likely represented by the Type I and II worms, respectively, based on the previous work of Rosen et al. (2000). Similarly, the 6-, 13-, and 20-day old worms in the 20-day experiment represented the Type I, Type II, and Type III worms, respectively (Rosen et al. 2000). Egg number increased and different egg developmental stages became apparent in the older experimental infections in our 20-day study (Figure 4).

No mortality or gradual progression of host pathology was noted in our timed (i.e., 7-, 14-, and 24-day) infections, and damage appeared to be restricted to the immediate area of worm attachment to the host mucosa. At the host-parasite interface, the fish mucosa was characterized by small lesions, and the mucosa and submucosa were constricted by the worm's oral sucker (Figure 5a). There was obvious epithelial necrosis and hemorrhaging at this attachment site (Figure 5b). Serial sections revealed complete detachment of host tissue (Figure 5c) undergoing advanced necrosis (Figure 5d) in the oral suckers of many worms. Notably, no experimentally infected fish died before they were sacrificed at the end of this study.

DISCUSSION

As predicted, based on this time frame for egg development (Rosen et al. 2000), it was possible to create an equal mix of *P. macrosotma* Type I and II and Type I to III adult worms in our 15- and 20-day laboratory experiments, respectively. This was accomplished by exposing a particular fish to equal numbers of cercariae at one or two weekly intervals. In natural infections, the proportions of these worm types within a host would thus provide an accurate measure of new infections with *P. macrostoma.*

Williams and Jones (1994) indicated that one of the most important factors affecting infection of hosts with parasitic worms is environmental temperature. Seasonal recruitment of trematodes lacking a metacercarial stage is clearly linked to such temperature changes. Several long-term studies with schistosomes have shown that maturation of cercariae within snails may slow or stop at low temperatures and that emergence itself may be inhibited (Schiff et al. 1975; Sinderman 1960). Lewis (1988) reported that *P. macrostoma* cercariae will not emerge from snails unless water temperature reaches 15°C, and Uglem (1980) recorded water temperatures at North Elkhorn Creek above 15°C only between March and October. Notably, Riley and Uglem (1995) observed no laboratory emergence of this species between October and

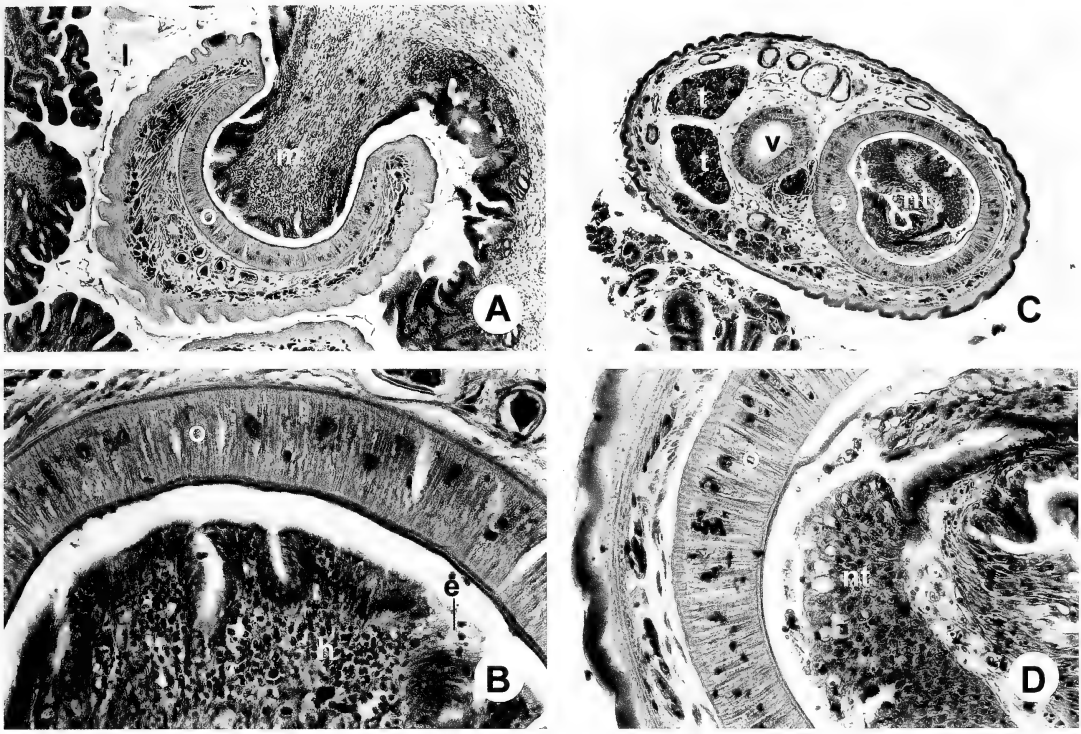

Figure 5. Histopathology of bluegill experimentally infected with *Proterometra macrostoma*: (A) worm oral sucker constricting host tissue, 100×; H & E (B) hemorrhaging and associated epithelial necrosis in constricted host tissue, 400×; H & E (C) detached host tissue in oral sucker of worm, 40×; Hematoxylin & Mallory's Trichrome (D) necrosis of detached host tissue, 400×; Hematoxylin & Mallory's Trichrome; e = epithelial necrosis, h = hemorrhage with erythrocytes, l = stomach lumen, m = host mucosa, nt = necrotic host tissue, o = oral sucker, s = host submucosa, t = testes, v = ventral sucker.

December in Kentucky. Thus it appears that *P. macrostoma* cercariae are available for infection of centrarchid fish at North Elkhorn Creek only from spring to early fall. The markedly high prevalence of Type I relative to Type II and III worms from naturally infected centrarchids during June and July in our study was likely correlated with increased maturation and shedding of cercariae during these months. Naturally infected fishes examined during the late fall to winter interval would likely possess only Type III or no worms because of (1) lack of cercarial availability due to water temperature and (2) loss of adults after 16 weeks (Riley 1992). A future, mid-winter collection of centrarchids would be required to verify this.

Procurement of nutrients by enteric, adult *P. macrostoma* is apparently accomplished by facilitated diffusion of sugars (Lewis and Uglem 1989) coupled with active ingestion of host tissues. With regard to the latter, histopathology in bluegill seemed limited to the area of worm attachment to the host mucosa. Apparently, these worms feed, detach, and move to other sites, leaving small but visible red lesions with associated hemorrhaging and epithelial necrosis. Overall damage was minor, and fish survival in both experimental and natural infections was unaffected.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Undergraduate Research and Creative Projects Program (URCPP) at Berea College to Ronald Rosen. We acknowledge the late Gary. L. Uglem, who provided the first author much insight and support with the *P. macrostoma* system. We thank Dr. Bernard Fried, Department of Biology, Lafayette College, and Dr. Allen Shostak, Department of Biolog-

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Development of the Human Mu, Kappa, and Delta Opioid Receptors and Docking with Morphine

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ABSTRACT

Opioid receptors belong to the superfamily of G protein coupled receptors and are primarily responsive to opiates to produce analgesia, but opiates also produce a variety of side effects. One goal of computational chemistry is to determine the interactions between a ligand and protein. This knowledge could allow for the development of opioid agonists without current side effects. Homology models of human mu, kappa, and delta receptors were developed based on a previously validated homology model of the endothelial differentiation gene. Docking of native ligand, morphine, was performed. The results indicate that the docking studies identified the actual active site in the model. Morphine had hydrogen bonds to Asp211, His361, and Ser381 in the mu receptor, and hydrogen bonds to Asp138 and His291 in the kappa receptor. Morphine had hydrogen bonds to Asp128 and His278 in the delta receptor. This correlates well with experimental data. We predict, based on the models, that mutation of Ser319 to alanine in the mu receptor would confer delta type binding. We further predict that mutation of Tyr312 to tryptophan in the kappa receptor would confer mu type binding. If Tyr312 were mutated to leucine, the resulting receptor would have delta type binding.

INTRODUCTION

G protein coupled receptors (GPCRs) comprise one of the largest receptor families. This family has over 1000 members and continues to grow (Klabunde and Hessler 2002). GPCRs mediate cellular responses including vision, chemotaxis, pain, allergy responses, and blood pressure. Pharmaceutical companies frequently target these receptors and current drugs target opioid, histamine, dopamine, and adrenergic receptors. Drug sales in 2000 for the top five drugs exceeded $10 billion (Klabunde and Hessler 2002).

GPCRs have the same molecular architecture and similar function to bovine rhodopsin (van Rhee and Jacobson 1996). The crystal structure was published in 2001 and elucidates the overall structure of this family (Palczewski et al. 2000). These receptors have seven transmembrane alpha helices that span the lipid bilayer. The amino terminus is extracellular and the carboxy terminus is intracellular. The agonist binding domain is within the helical bundle (van Rhee and Jacobson 1996).

Opioid receptors, one GPCR subclass, are divided into three subclasses, the mu, kappa, and delta. These divisions are based on pharmacology and physiology (Raynor et al. 1994). The three receptors have different affinity for morphine (Ki values are mu 6.55 nM, kappa 113 nM, and delta 217 nM) (Lattanzi et al. 2005). If the differences in affinity are based on differences in the binding site, computational methods could be used to determine these differences.

Morphine, an opiate that causes analgesia, can be used for management of pain. Unfortunately, morphine also has side effects including respiratory depression and decreased gastrointestinal motility (Raynor et al. 1994). This alkaloid is also subject to abuse since euphoria is associated with its use. The development of an opioid receptor agonist that relieves pain without side effects and that is not subject for abuse has long been sought (Kieffer and Evans 2002; Stevens 1994). One step in that process is to understand the molecular interactions between the ligand and the receptor.

The focus of our study was the development of the mu, kappa, and delta opioid receptors and the docking of those models with morphine. Interactions between the receptor and ligand were analyzed to select the best complex. These complexes were subjected to molecular dynamics to test the stability of the complex. The receptor/ligand interactions

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Table 1. Accession numbers for opioid receptor sequences used in this study.

Chain	Receptor	Sequence accession number
1	Human Mu	NP_000905
2	Mouse Mu	NP_035143
3	Rat Mu	NP_037203
4	Human Kappa	NP_000903
5	Mouse Kappa	NP_035141
6	Rat Kappa	NP_058863
7	Human Delta	NP_000902
8	Mouse Delta	NP_038650
9	Rat Delta	NP_036749
10	EDG1 Model	

were compared to experimental literature to verify the accuracy of the models. Finally, the docking results were used to predict what residues were needed for binding, helical packing, and binding profiles.

MATERIALS AND METHODS

Alignment

All modeling in this study was completed using the Molecular Operating Environment (MOE) software package developed by the Chemical Computing Group Inc. (MOE 2003). The sequences downloaded from National Center for Biotechnology and Information (NCBI) are listed in Table 1 and represent human, mouse, and rat sequences for the mu, kappa, and delta receptors. The sequences were aligned with the template structure, the previously published EDG1 model (Bautista et al. 2000; Fischer et al. 2001; Parrill, Baker et al. 2000; Parrill, Wang et al. 2000; Sardar et al. 2002; Wang et al. 2001) using the default settings in MOE. Homologies of the opioid sequences, based on identity, ranged from 94% to 47%. The homology of the template was much lower and ranged from 22% to 20% (Table 2). This low homology would generally be of concern, but GPCR homology models have been successfully developed with homology in this range (Klabunde and Hessler 2002). The most conserved residue was aligned manually for each helix (van Rhee and Jacobson 1996) (Figure 1).

Models

Homology models of the human mu, kappa, and delta receptors were developed using the default settings in MOE. The template selection is critical for model development. The template should share significant sequence homology and have similar function (Klabunde and Hessler 2002). Two main template sequences often used are the crystal structure of bovine rhodopsin [PDB ID 1F88] (Palczewski et al. 2000) and a theoretical model of bovine rhodopsin based on electron microscopy [PDB ID 1B0J] (Pogozheva et al. 1997). The crystal structure is considered an inactive template because it was generated in the dark (Bissantz et al. 2003; Palczewski et al. 2000). A third template, endothelial differentiation gene 1 (EDG1), was used in our study. EDG1 is a GPCR whose function is to act as a receptor for phospholipids (Bautista et al. 2000). The EDG1 model was developed based on the 1BOJ template with modifications (see Bautista et al. 2000). This template was selected since it has the extracellular and intracellular loops unlike 1BOJ that is compromised of only the transmembrane helices and the model was experimentally validated.

The models were minimized to a root mean squared gradient (RMSG) of 0.1 Kcal/mol·Å

Table 2. Sequence homology for alignment of the human, mouse, and rat opioid sequences. Chains are listed in Table 1.

Chains	1	2	3	4	5	6	7	8	9	10
1		90.5	94.0	60.5	59.7	60.3	58.6	58.3	59.1	20.6
2	78.6		95.0	60.0	59.5	59.7	58.3	57.8	58.6	19.9
3	81.0	94.3		60.3	59.7	60.0	58.1	57.8	58.6	19.9
4	49.8	56.9	57.5		93.7	94.2	58.9	58.1	58.3	19.6
5	49.1	56.4	57.0	93.7		98.9	57.8	57.3	57.5	19.9
6	49.6	56.6	57.3	94.2	98.9		58.3	57.8	58.1	19.9
7	47.2	54.1	54.3	57.6	56.6	57.1		93.0	93.5	22.3
8	47.0	53.6	54.0	56.8	56.1	56.6	93.0		96.5	22.3
9	47.6	54.4	54.8	57.1	56.3	56.8	93.5	96.5		22.3
10	13.4	15.0	15.1	15.5	15.8	15.8	18.0	18.0	18.0	

using the Amber94 force field (Cornell et al. 1995). A protein report was generated to determine if any angles or dihedrals exceeded biochemical norms (outliers). All outliers were in loop regions, which were ignored and not used in this study.

Each model was then used for docking of morphine. A model of morphine was built in MOE and the nitrogen was protonated to represent the structure at physiological pH. Chirality of the centers was determined and maintained in the model. The resulting structure was then minimized in Merck Molecular force field (MMFF94) (Halgren 1996).

Docking

Morphine was docked into the mu, kappa, and delta receptors. Docking was accomplished using the algorithm MOE Dock. The default settings were used with the exception of Tabu search. A Tabu search will disallow a previous docking location for subsequent docking runs to ensure that the entire docking volume is probed. For each position, 700 iterations were generated to optimize the interactions at a location. A database of 25 complexes for each receptor with the ligand was generated. MMFF94 force field (Halgren 1996) was used. Each complex was visually inspected to determine if the ligand was in the upper one-third of the receptor and inside the helical bundle. Hydrogen bonds between the ligand and receptor and aromatic residues were determined. Based on these criteria, the best complexes of each were selected and then minimized to an RMSG of 0.1 Kcal/mol·Å using the MMFF94 force field. Each complex was checked to determine if hydrogen bonding had increased, decreased, or remained the same. Any changes in aromatic interactions were also noted.

Dynamics

The best complex for each ligand and receptor was then subjected to unrestrained molecular dynamics simulations. Dynamics were performed on all three complexes generating molecular databases. This calculation used NVT parameters (holding constant moles, volume, and temperature). The simulation was performed for 1 fs time step with 60 ps of heating prior to the 100 ps equilibrium (data collection) phase as in Wang et al. 2001. The output databases contained 100 entries collected during the equilibrium phase. The simulation calculated potential energy (U in Kcal/mol), temperature (T in kelvins), pressure (P in Kpa), total energy (E, kinetic and potential in Kcal/mol), and enthalpy (H, E + PV in Kcal/mol).

RESULTS

Mu Model

The mu model was minimized to an RMSG of 0.1 kcal/mol·Å. The protein report indicated five cis amide bonds all located in extra- or intra-cellular loops. Since these locations were not used in the results, the amide bonds were not corrected. The helices were between 33 and 20 residues in length, with helix 5 being the shortest (Table 3). This helix, in all three receptor models, was terminated early on the intracellular side. This area is indicated in activation and not in ligand binding and was therefore ignored.

Kappa Model

The kappa model was minimized to an RMSG of 0.1 kcal/mol·Å. The protein report indicated three cis-amide bonds and all were located in loop regions. Since these locations were not used in any docking results, the amide bonds were not corrected. The helices varied in length from 20 to 33 residues. Helices 4 and 5 were both 20 residues long, unusually short for spanning the lipid bilayer. Helix 4 is terminated by P172 located at the intracellular surface. As in the mu receptor, the kappa receptor helix 5 is terminated early at the intracellular surface (Table 3).

Delta Model

The delta model was minimized to an RMSG of 0.1 kcal/mol·Å. The protein report indicated three cis-amide bonds and all were located in loop regions. Since these regions were not used in the docking results, the amide bonds were ignored. The helices varied in length from 19 to 29 residues (Table 3). Again, helix 5 was terminated at the intracellular surface. Helix 4 was also terminated at the intracellular surface by Pro162.

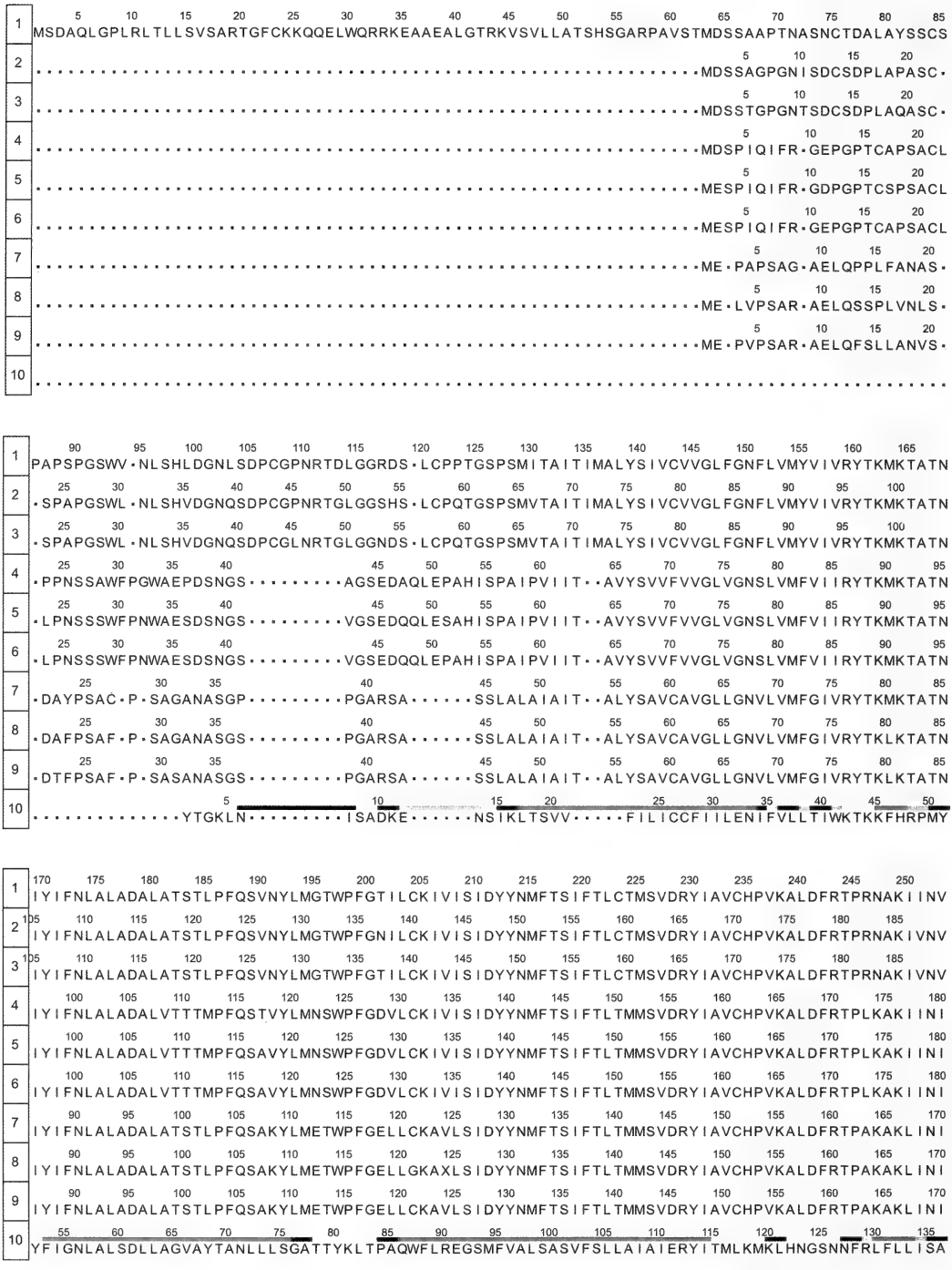
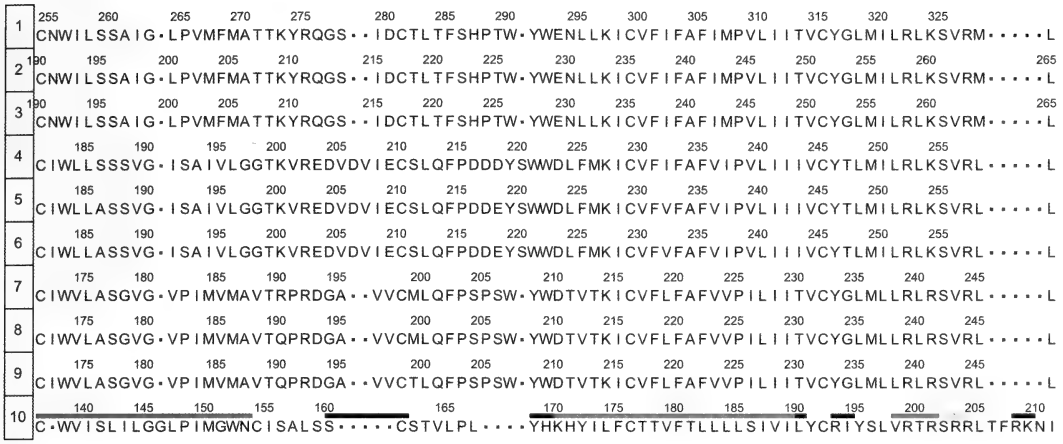


Figure 1. Alignment of opioid sequences for human, mouse, and rat with EDG1 model. See Table 1 for chain assignments. Continued on next page.



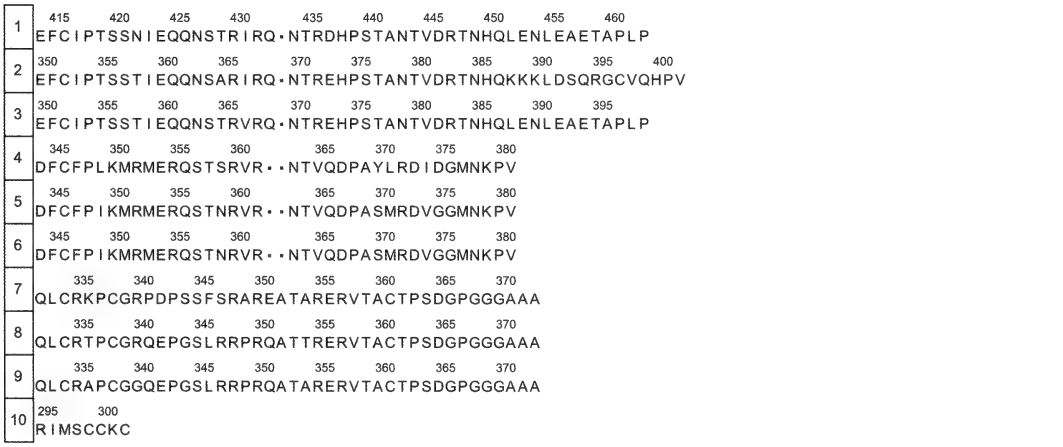


Figure 1. Continued.

Table 3. Residues involved in helices 1–7 in the opioid receptors.

Helix	Mu receptor	Kappa receptor	Delta receptor
1	P127-V158	I54-F82	A47-V75
2	Y170-Y192	T94-Y119	T84-Y109
3	L203-I231	G127-I158	L120-148
4	T244-A270	A174-A193*	L167-V188**
5	L295-V314**	F225-V244**	S204-V233***
6	L339-I366	R270-V296	R258-V283
7	T379-F411	A308-F341	L302-K326

* P172 terminates this helix.
** P162 terminates this helix.
*** Helix is terminated early on the intracellular surface.

Docking

Morphine was docked into the three receptor models mu, kappa, and delta. The resulting databases were evaluated visually based on placement in the helical bundle, the number of hydrogen bonds to side chains of the receptor, and the number of aromatic residues within 3 Å. The best complex was then minimized to an RMSG of 0.1 Kcal/mol·Å using the MMFF94 force field. The results for each receptor are listed in Table 4.

Mu with Morphine

Of the 25 complexes for mu with morphine, 12 had hydrogen bonds between the ligand and the receptor. Four of these complexes were rejected due to position in the receptor. Of the eight remaining, one had hydrogen bonding to two side chains of the receptor: His361 and Asp211. Phe301 and Trp382 were within 3 Å of the ligand (Figure 2; Table 4). All other docking runs had only one hydrogen bond to the receptor.

Kappa with Morphine

Of the 25 docking complexes, only 12 had hydrogen bonding to the receptor. Of the 12 complexes, eight were eliminated due to position in the receptor. Of the four remaining complexes, three had hydrogen bonds to the side chains of the receptor. All of these complexes were minimized to an RMSG of 0.1 Kcal/mol·Å. One of these three increased hydrogen bonding to two side chains: Asp138 and His 291. Aromatic residues within 3 Å of the ligand were Tyr139, Phe231, Trp287, and Tyr312 (Figure 3; Table 4).

Delta with Morphine

Of the 25 docking runs, six had hydrogen bonding between the receptor and the ligand. Of these, only three were within the helical bundle. All three were minimized to an RMSG of 0.1 Kcal/mol·Å. One increased hydrogen bonding to two side chains: Asp128 and His278. Only Tyr129 was within 3 Å of the ligand (Figure 4; Table 4).

Dynamics

Each complex was subjected to 100 ps of unrestrained molecular dynamics under NVT conditions. The resulting databases contained 100 entries collected over the equilibrium phase. The simulation calculated potential energy (U in Kcal/mol), temperature (T in kelvins), pressure (P in Kpa), total energy (E, kinetic and potential in Kcal/mol), and enthalpy (H, E + PV in Kcal/mol). The average, standard deviation and percent standard deviation was calculated for the three databases. The percent standard deviation for each parameter varied from 0.9% to 1.8%. The databases were also visually inspected. The complexes would momentarily lose tertiary structure but would regain that structure in subsequent database entries. The delta/morphine complex maintained the same hydrogen bonds throughout the course of the simulation. The kappa/morphine complex would vary a hydrogen bond between His291 and Cys315. The hydrogen bond/ion pair with Asp138 was in every entry in the database. The mu/morphine simulation

Table 4. Summary of docking results for morphine and all opioid receptor models. K_i values are in nM from Lattanzi et al. 2005. sc indicates side chain interaction, bb indicates backbone interaction.

Receptor	K_i*	Hydrogen Bonding Residues	Aromatic Residues
Mu	6.55	Asp211 sc, His361 sc, Ser381 bb	Phe301, Trp382
Kappa	113	Asp138 sc, His291 sc, Cys315 sc (in some dynamics complexes)	Trp287, Phe231, Tyr139, Tyr312
Delta	217	Asp128 sc, His278 sc	Tyr129

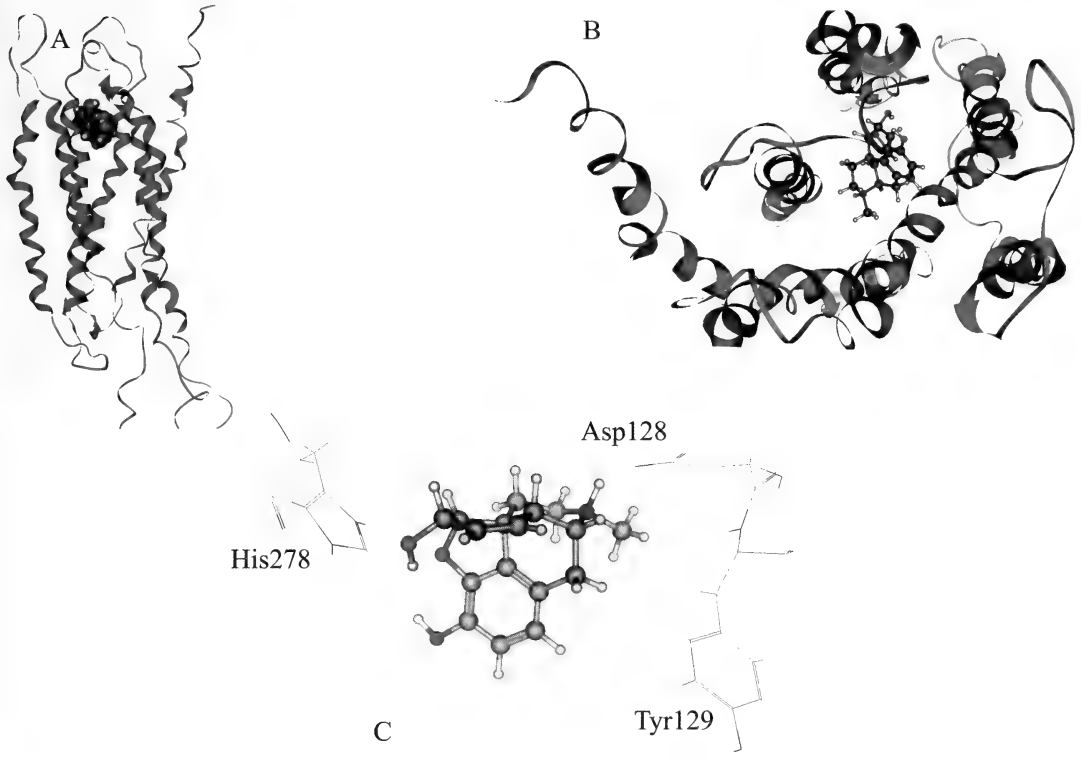


Figure 2. Complex of morphine with the mu model. Panel A, view from the side showing placement of ligand in the receptor. Panel B, view from extracellular space showing placement of ligand in the helical bundle. Panel C, close-up of ligand and residues in the receptor. Hydrogen bonds are shown as dotted lines.

had the same hydrogen bonds listed and, in addition, a hydrogen bond to the side chain of Ser381 in some but not all entries of the database. Overall, the complexes, very stable, maintained the same network of hydrogen bonds.

DISCUSSION

To evaluate the fitness of the models, they were compared to experimental data in the literature. The literature review was not exhaustive but surveyed some of the more important findings of others to determine if the receptor models developed in our study were in accordance with experimental data.

In one computational study of the human opioid receptors, ab initio models were developed (Strahs and Weinstein 1997). These models were developed before the crystal structure of bovine rhodopsin was solved and therefore used ideal helices and packed them based on the diffraction map of bovine rhodopsin (Schertler et al. 1993). The helices were oriented based on the calculated hydrophobic moment that oriented the most hydrophobic side to the lipid bilayer. The residues Fukuda et al. (1995) predicted to be inward facing and that varied across the opioid receptors are listed in Table 5. They reasoned that since the position varied and it was predicted to be inward facing, it might be involved in ligand selectivity. Of the 14 residues listed, only three are inward facing in our models: Ala181, Val364, and Trp382. Many are oriented towards other helices or the lipid bilayer. The validity of using a hydrophobic moment calculation can be questioned since the data presented by Befort et al. (1999) disagree with models based on experimentally validated models. Of the three residues that are inward facing only one is largely different. The variation at Ala181 is minimal, either alanine or valine. Neither substitution would greatly impact either charge or size at that position and would therefore not account for binding difference in the opioid family. Val364 is likewise

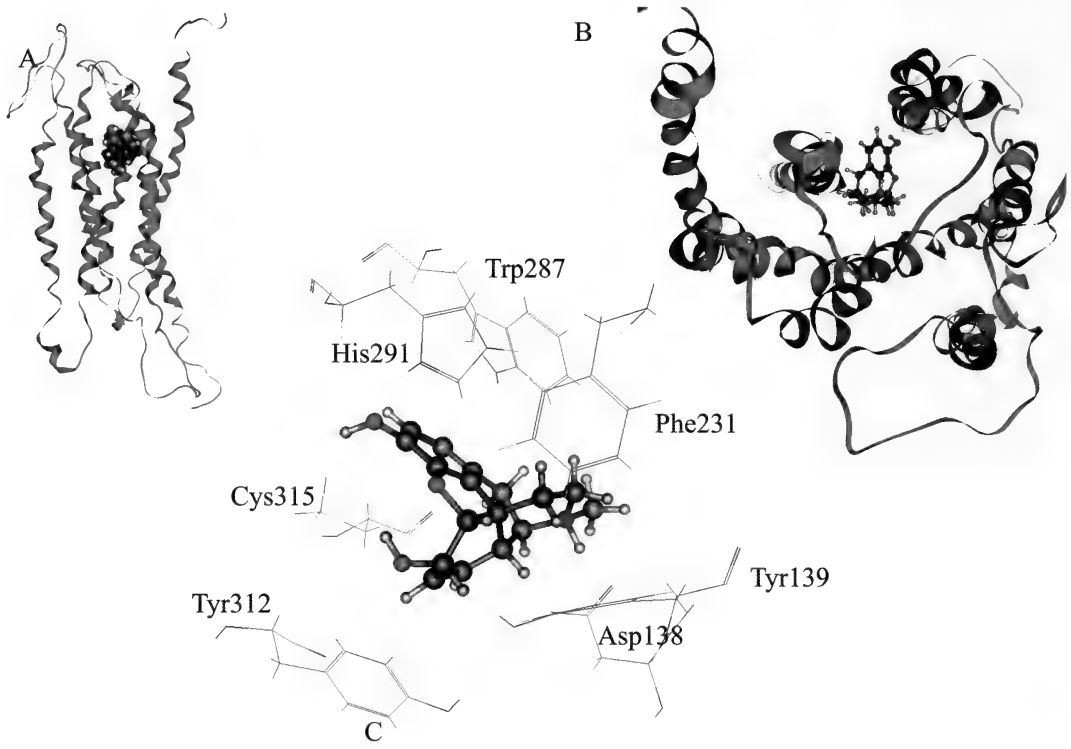


Figure 3. Kappa receptor with morphine. Panel A, view from the side showing placement of ligand in the receptor. Panel B, view from extracellular space showing placement of ligand in the helical bundle. Panel C, close-up of ligand and residues in the receptor. Hydrogen bonds are shown as dotted lines.

a small variation, either valine or isoleucine. Again, neither residue would produce a large change in size or charge. However, Trp382, in mu, is either Tyr312 in kappa or Leu300 in delta. This mutation changes the size and charge at this location. The kappa and mu complexes both have the residue within 3 Å of morphine. The variance between our model and the model by Strahs and Weinstein (1997) might be due to the projection map of bovine rhodopsin and how the helices were oriented by these researchers.

In another study, a series of chimeric receptors was expressed and binding of morphine was determined by Fukuda et al. (1995), who reported that helices 5–7 were needed for mu type binding. In our study, we found that morphine binds to residues in helices 3 (Asp211), 6 (His361) and 7 (Ser381). The residues in helices 3 and 6 are found in all three receptor complexes at the same location and therefore were not indicated as selective for one receptor. However, we found a large variation of aromatic interactions for the three receptors. We propose that these differences do account for the differences in the binding profiles in the opioid receptors. The mu/morphine complex has aromatic interactions with helices 5 and 7. The kappa/morphine complex has aromatic interactions with helices 3, 5, 6, and 7. Finally the delta/morphine complex only has aromatic interactions with helix 3. These results indicate that the model agrees with the mutagenesis of Fukuda et al. (1995).

A mutagenesis study by Befort et al. (1996) examined the role of aromatic transmembrane residues to determine the role these residues played in the binding of various ligands and the delta opioid receptor. They concluded that Try129 contributed a large part of the ligand binding in the mouse delta opioid receptor. They further stated that there is likely a general binding domain but that each ligand will bind uniquely. In our work, we found that the three receptor models all had interactions with morphine and aspartic acid in helix 3 and his-

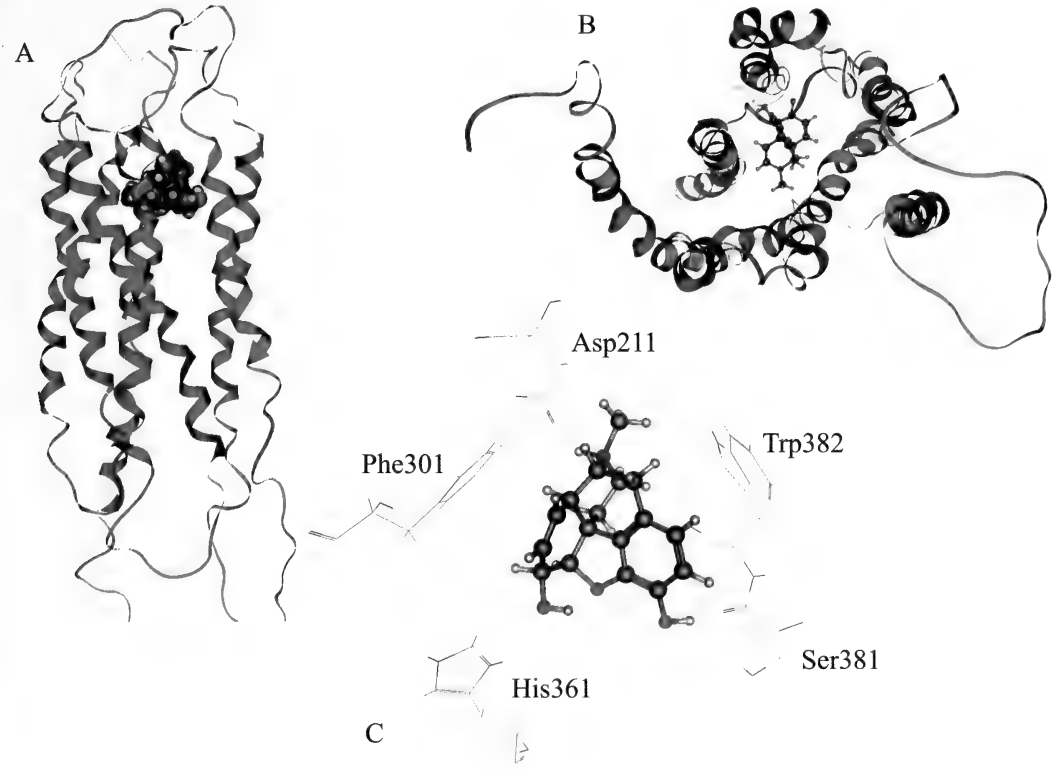


Figure 4. Delta receptor with morphine. Panel A, view from the side showing placement of ligand in the receptor. Panel B, view from extracellular space showing placement of ligand in the helical bundle. Panel C, close-up of ligand and residues in the receptor. Hydrogen bonds are shown as dotted lines.

Table 5. Analysis of data presented by Strahs and Weinstein 1997. Residues listed are in the human mu opioid receptor. Residues that the mu model indicates are inward facing are in bold.

Helix	Residues	Orientation
1	Met136	Towards other helices
	Csy143	Towards other helices
2	**Ala181**	**Inward facing**
	Leu185	Towards other helices
	Asn191	Outward facing
3	Ile208	Outward facing
4	Leu264	Outward facing
5	Ile302	Towards other helices
6	**Val364**	**Inward facing**
	Lys367	Extracellular surface
7	Gln378	Extracellular surface
	Thr379	Extracellular surface
	Trp382	**Inward facing**
	His383	Towards other helices

tidine in helix 6. We also noted that Try129 in the delta receptor was within 3 Å of morphine.

In a second study, the same group investigated the importance of residues Asp128, Tyr129, and Tyr308 in the mouse delta opioid receptor (Befort et al. 1999). They found that mutation of any of these three residues caused an agonist independent activation of the receptor. In our study, Asp128 is involved in hydrogen bonding with morphine. We also found that Tyr129 was within 3 Å of morphine. Tyr308 (also 308 in human delta receptor) appears to be involved in helical packing and interacts in helix 1 with Tyr56, Val59, and Gly63, and in helix 2 with Asp94, Ala98, and Thr99. The mutation of this residue would affect the packing of the helical bundle and could result in constitutively active receptor.

A site-directed mutagenesis study (Mansour et al. 1997) attempted to determine the residues lining the binding site of the mu opioid receptor. Two key residues were indicated to

decrease the binding of morphine in the rat mu receptor, Ile198 (262 in the human) and Tyr326 (390 in the human). From our models, we propose that these residues are needed for helical packing and not for binding. Ile262 in helix 4 interacts with Tyr212 (helix 3) and Val300 (helix 5). Tyr390 interacts with Val142, Cys143, Gly146, all in helix 1, and Asp178, Ala181, and Thr182 in helix 2.

In a study of the rat mu receptor, Bot et al. (1998) mutated residues that in our study are directly needed for ligand/receptor interactions. They mutated Asp114 to asparagine and binding of a variety of ligands decrease from 8 to 41 fold. Agonist and peptide agonist were affected but partial agonists and antagonists were not. They also mutated His297 to asparagine however this mutation was less effective in reducing binding affinity of agonists but did affect partial agonists and antagonists. Morphine binding was decreased by two fold. These two residues are indicated in the binding of morphine in all three models.

Based on the above studies, the models presented here are in good correlation with experimental data and are expected to have good predictive ability. Our models predict that in the mu receptor, Ser319 mutation to alanine would confer delta type binding. If Tyr312 of the kappa receptor were mutated to tryptophan, the resulting receptor would have a mu type binding profile. If the same residue (kappa Tyr312) were mutated to leucine, the resulting receptor would have delta type binding.

SUMMARY

The models presented here are in good agreement with experimental literature. The models identified an aspartate and a histidine that are required by all three receptors for binding of morphine. The models also identified residues that if mutated could impart a different binding profile. The models were also in agreement with mutagenesis data regarding two residues required for binding, an aspartic acid in helix 3 and a histidine in helix 6, and two residues required for helical packing, tyrosine in helix 7 and an isoleucine in helix 5. While these models represent only the active state of the receptor, it does offer some insight into an important biological process.

ACKNOWLEDGMENTS

Support received from the NIH/NCRR/, Grant # 1 P20 RR16841-01 and by NSF KY EPSCoR REG is gratefully acknowledged.

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Salicylate Inhibits Growth of Plant-Pathogenic Fungi and Synergistically Enhances the Activity of Other Antifungal Materials *In Vitro*

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ABSTRACT

We conducted dose-response studies of the toxicity of salicylate (SA; a putative signal molecule for enhancement of plant disease resistance [EPDR]) toward plant-pathogenic fungi. SA concentrations ≥10.0 mM were required for complete inhibition of fungal growth from mycelial plugs. SA doses of 2.0–5.0 mM typically reduced fungal growth by 50%, whereas doses of 0.5 mM or lower had little or no effect on fungal growth. However, growth of several test fungi was completely inhibited by 2.0 mM SA combined with concentrations of cupric chloride, antifungal bacterial culture fluids, or neem extract that were otherwise only slightly to moderately inhibitory. We conclude that (1) endogenous SA concentrations (up to 10.0–100.0 μM) are unlikely to directly inhibit fungi in plants, (2) concentrations of exogenous SA applied for EPDR (2.0–10.0 mM) are likely to be only moderately inhibitory to fungi, and (3) additions of other antifungal materials with which SA synergizes may enhance the antifungal activity of SA applied to plant surfaces for EPDR. The latter conclusion provides a rationale for further study of the synergistic interactions of moderately active antifungal materials for practical plant disease control.

INTRODUCTION

Salicylate (SA) has been intensively studied as a putative endogenous signal molecule for the activation of plant defenses against pathogens. Total endogenous SA may increase to perhaps 10.0–100.0 μM (locally) in infected plant tissues, whereas 2.0–10.0 mM exogenous SA is commonly applied to plants as a foliar spray for the experimental enhancement of plant disease resistance. The large discrepancy between the concentrations of endogenous or exogenous SA required for activation of plant defenses has been discussed (Norman et al. 2004; Shirasu et al. 1997). The lower concentrations of endogenous SA may act via synergistic enhancement of the generation of reactive-oxygen species (ROS) that occurs upon exposure of plants to pathogens or pathogen-derived molecules (the "oxidative burst") (Shirasu et al. 1997), and/or may trigger the expression of an alternate oxidase in plant mitochondria that results in reduced susceptibility of plant tissues to damage by ROS during pathogenesis (Xie and Chen 1999). The higher concentrations of exogenous SA used to activate disease resistance have been demonstrated to inhibit plant catalase activity, and the resultant accumulation of hydrogen peroxide in plant tissues has been proposed to be a trigger for the activation of anti-pathogen defenses (Chen et al. 1993). Ruffer et al. (1995) challenged this concept based on several lines of evidence, including their finding that SA can inhibit a variety of enzymes of plant, animal, and fungal origin, and the fact that concentrations of endogenous SA comparable to those required to inhibit plant catalase occur seldom, if ever, in plant tissues. Ruffer et al. (1995) also proposed that rather than serving as a specific signal for activation of plant defenses, SA may simply function as a phytoalexin (an inducible low-molecular weight antimicrobial compound made by plants in response to pathogen invasion or certain other stressors). Salicylate also is well known as an uncoupler of mitochondrial electron transport and chemiosmosis (Norman et al. 2004), and has been used as an antimicrobial food additive (Cruess and Irish 1931). Thus, we hypothesized that SA might also be directly inhibitory to plant pathogens via one or more such mechanisms, particularly at the millimolar concentrations a pathogen might encounter on a plant leaf that has been sprayed with exogenous SA to activate plant defenses. We tested this hypothesis with in vitro studies of the effects of SA on the growth of seven plant-pathogenic fungi. Further, we experimentally

Table 1. Sources and sensitivities toward salicylate (SA) of the plant-pathogenic fungi employed. Organismal names are presented as genus and species, with abbreviation employed in the text. The sources listed indicate the diseased plant material from which fungi were isolated. Assays were of mycelial growth from mycelial plugs (MG), spore germination (SG) or mycelial growth from spores (MGS) conducted in potato-dextrose agar (PDA) in standard 15 × 100 mm Petri dishes or in a liquid glucose-salts medium (GS) in 24-well plates. IC_{100} and IC_{50} values represent the concentrations of SA found to inhibit MG, SG, or MGS by either 100% or 50%, relative to controls not exposed to SA. Ranges of values indicate the tested SA concentrations between which interpolated IC values logically fall.

Organisms	Sources	Assay	IC_{100} (mM SA)	IC_{50} (mM SA)
Botrytis cinerea (BC)	Rose (flower) Petal blight	MG/PDA	10.0–20.0	2.0–5.00
Colletotrichum graminicola (CG)	Corn (leaf) Anthracnose	SG/GS	0.75–1.0	0.5–0.75
Dendrophoma obscurans (DO)	Strawberry Leaf blight	MG/PDA	5.0–10.0	1.0–2.0
Diplocarpon rosae (DR)	Rose (leaf) Black spot	MG/PDA	>10.0	1.0–2.0
Monilinia fructicola (MF)	Peach (fruit) Brown rot	MGS/GS	≤10.0	2.0–5.0
Pestalotia sp. (PESP)	Grape (leaf) associated with Black rot lesions	MG/GS	10.0	2.0–5.0
		SG/GS	>2	>2
Pythium sp. (PYSP)	Turf Web blight	MG/PDA	>20.0	5.0–10.0

evaluated the hypothesis that SA might synergistically enhance the activity of other antifungal materials. Although SA has been reported to synergistically enhance plant responses to molecules that elicit the expression of disease resistance mechanisms (Shirasu et al. 1997), there appear to be no previously published reports of synergism of SA with other antifungal materials, other than our own abstract (Strobel and Porter 2005) of a poster presented at the 2004 Annual Meeting of the Kentucky Academy of Science. Synergistic interactions between conventional fungicides have been reported (Kosman and Cohen 1996).

MATERIALS AND METHODS

Culture Media and Related Materials

Deionized or double-distilled water were used throughout these investigations. Potato-dextrose agar (PDA) and potato-dextrose broth (PDB) were purchased from Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60103. Sodium salicylate (SA; USP grade) was purchased from Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178. Glucose was purchased from Mallinckrodt Baker, Inc., P.O. Box 800, Paris By-Pass, US 68, Paris, KY 40361. Mineral salts were of ACS grade or higher, and were purchased from Fisher or Sigma. One liter of the liquid glucose-salts (GS) medium we employed contained the following ingredients: glucose, 10 g; with macronutrients NH_4NO_3, 700 mg; KH_2PO_4, 1200 mg; $MgSO_4{\cdot}7H_2O$, 300 mg; $CaCl_2{\cdot}2H_2O$, 300 mg; and the following micronutrients: $MnCl_2{\cdot}4H_2O$, $FeCl_3{\cdot}6H_2O$, $ZnSO_4{\cdot}H_2O$, and $CuSO_4$, 80 μg each. Stocks of each mineral nutrient (macronutrients, 100×; micronutrients, 1000×) were autoclaved separately, as were glucose solutions. A commercially-formulated herbicide was used as our paraquat (PQ) source (Gramoxone Extra, 2.5 lbs of PQ per gallon, Syngenta Corporation, 2200 Concord Pike, P.O. Box 8353, Wilmington, DE 19803). Two commercially available neem (NM) preparations, both listing clarified hydrophobic extract of neem oil as the active ingredient (a.i.), were purchased locally. These were Green Light Tomato and Vegetable Spray, Ready-To-Use, containing 0.9% a.i. (NMRTU; Green Light Company, San Antonio, TX 75418) and Garden Safe® Fungicide 3™ Concentrate containing 70% a.i. (NMC; Schultz Company, Bridgeton, MO 63044). Polystyrene Petri plates (100 × 15 mm) and polystyrene 24-well BD Falcon culture plates were purchased from Fisher. Fluorescent lighting tubes were purchased locally. The germicidal ultraviolet lamp (model UVG-54) employed for visualization of SA fluorescence was purchased from UVP, Inc., 2066 W. 11th Street, Upland, CA 91786.

Characteristics, Sources, and Maintenance of Microorganisms

The names, in-text abbreviations, characteristics, and sources of the microorganisms employed in this research are presented in Table 1. We isolated all fungi (except *Colletotrichum graminicola*, CG) from naturally-infected plant materials collected in the area of Lex-

ington, KY. Fungi were maintained by serial transfer on PDA, typically at 1–4 week intervals, although sometimes more frequently. Stock and experimental cultures were maintained at room temperature, typically 20–22°C. *Botrytis cinerea* (BC) and *Pestalotia* sp. (PESP) stock cultures were commonly maintained in the dark, although PDA cultures of PESP and CG were sometimes maintained under standard fluorescent lights (12 hr light/dark and 24 hr light, respectively) to promote spore germination. Spore suspensions of CG and PESP were prepared by agitating small plugs (ca. 3 × 10 mm) cut from sporulating cultures on PDA in sterile distilled water (SDW) with a vortex mixer. Spores of *Monilinia fructicola* (MF) were produced by culturing the fungus on commercially-available unsulphured dried apricots, which were rehydrated prior to sterilization by autoclaving. Spores of MF were transferred to a small volume of SDW with a transfer loop, and the suspension was agitated with a vortex mixer. Because the presence or absence of light was otherwise found to have no effect on the outcomes of our experiments, inoculated plates were maintained under either condition, as space permitted.

Two mixed cultures of bacteria that exhibited antifungal activity in preliminary co-culture experiments with BC and PESP were designated as "brown" or "white" based on their appearance in PDA culture. The brown culture was isolated from rose leaves exhibiting symptoms of the black spot disease (caused by *Diplocarpon rosae*, DR) and was found to completely prevent DR spore germination on PDA when present in inoculum drops. This brown culture begins to produce a visually detectable, brown diffusible pigment 3–4 days after inoculation to PDA or PDB culture media. The white bacterial culture was obtained as a laboratory contaminant. It also appeared to produce one or more diffusible colorless antifungal substances on PDA. Bacterial culture fluids (BCF) from prolonged (18 days) shake cultivation of the brown and white cultures in PDB (designated BRCF and WCF, respectively) were employed. This lengthy cultivation period appeared to have resulted in the death of bacteria, based on the absence of growth from aliquots of culture fluids plated onto PDA. Bacterial cultures were maintained by serial transfer on PDA, usually at 1–4 week intervals.

Assessment of SA Effects on Growth of Fungi on PDA

Because SA is not stable at autoclave temperatures, culture media were amended with SA after autoclaving. Petri plates containing ~20 ml of PDA were amended with 200 µl of SDW or 200 µl of sterile aqueous stock solutions of SA applied to the solidified PDA and allowed to diffuse evenly throughout the agar for 3–4 days. The extent of SA diffusion in these plates was assessed by brief visualization of SA fluorescence under a germicidal UV lamp. Plugs (typically 3–5 mm on a side) were cut from the margin of 3–5 day fungal cultures on PDA and placed in the center of Petri plates. The resultant fungal colonies were measured when the fastest-growing colonies neared the edges of Petri plates, often after 2–3 days incubation at 20–22°C in the dark. Long and short diameters of the typically ovoid individual colonies were averaged for statistical analyses.

Detection and Assessment of Synergistic Interactions of SA with Other Antifungal Materials

Dose-response studies with individual materials were conducted with each of the test fungi employed in these experiments (BC, CG, and PESP) to guide the selection of suitable doses for interaction studies. For experiments conducted on PDA, SA and PQ were added after autoclaving. For experiments conducted in 24-well culture plates, aliquots of the liquid GS medium (0.4, 0.5, or 1.0 ml for mycelial growth assays, 0.4 ml for spore germination assays) were added to wells and then amended with stock solutions of antifungal materials. In studies of the interactive effects of SA and PQ on BC growth in well-plate culture, incubation continued until wells that supported significant growth of BC were filled with fungal mycelia (typically 15 days). Growth was assessed visually and evaluated as present/strong (+), absent (−), or weak (±). In studies of SA interactions with Cu or BCF in 24-well plates, fungal growth in well-plate assays was assessed by several techniques, usually after 3–4 days for mycelial growth assays and after 1 day for spore germination assays.

The presence or absence of growth was first assessed visually, and absence of growth then verified by microscopic observation. Radial mycelial growth from agar plugs was assessed visually with a millimeter rule, by viewing plates from beneath by transmitted ceiling light, or sublit on a microscope stage upon which the rule had been placed. In some experiments ("NMRTU time-course studies"), addition of SA and inoculation with fungi were delayed relative to introduction of NMRTU. Mycelial growth of BC in the NMRTU time-course study presented in this paper was evaluated relative to that of controls according to the following rating system: 0 = no growth; 1 = >90% inhibition (weak growth); 2 = 75% inhibition (weak-moderate growth); 3 = 50% inhibition (moderate growth); 4 = 10–25% inhibition (moderate-strong growth); 5 = 90–100% of maximal growth (strong growth). Germination of CG and PESP spores was assessed with a microscope at 100× total magnification. In experiments with CG spores, a minimum of 120 spores per well were evaluated.

The nature of interactions between SA and these other antifungal materials was determined according to the method of Kosman and Cohen (1996). For example, in Table 3, the observed means of various treatments involving doses of single agents (such as 2.0 mM SA or 4.0 or 8.0 mM PQ) are first converted into percentages of the observed mean values (OPCs) for controls (which received neither SA nor PQ). Then a simple additive model of interaction (AMI) is employed to calculate the values (predicted percent of control values, PPC) one would expect to see (in the absence of either antagonistic or synergistic interactions between test compounds). The additive model assumes the independent impact of each agent on a test organism (such as the fungus, BC), and the impact of two agents together is calculated as the product of the impact of the first times the impact of the second. Thus, in Table 3, we find that 2.0 mM salicylate alone reduced BC growth to 80% of the water controls, whereas 4.0 mM PQ reduced BC growth to 31% of the water controls. The PPC predicted by the AMI for the combination of 2.0 mM SA and 4.0 mM PQ is determined by multiplying 0.80 times 0.31, which yields a PPC of 0.25, or 25%. An observed percent of control (OPC) value larger than 25% would indicate a lesser degree of inhibition than predicted by a simple additive model of interaction, and would constitute evidence of an antagonistic (ANT) interaction between the two test substances. In the present example, the OPC for the SA-PQ combination mentioned above (12%) is lower than that predicted by the AMI (25%), and the interaction is thus judged to have been synergistic (a greater than predicted or expected degree of inhibition of BC by the SA-PQ combination was observed).

RESULTS

Dose-Dependent Inhibition of the Growth of Plant-Pathogenic Fungi by SA

A summary of the sensitivities of BC and other fungi to SA added to PDA or GS is presented in Table 1. SA concentrations comparable to those that may occur in infected plant tissues (10.0–100.0 μM endogenous SA, data not shown) had no observable effect on BC mycelial growth in our experiments. Rather, SA concentrations required for complete inhibition (IC_{100}) of mycelial growth on PDA or GS media were typically ≥10.0 mM, whereas between 1.0 and 5.0 mM SA were required to inhibit mycelial growth on PDA by 50% (IC_{50}) for most fungi, and the IC_{50} of PYSP was 5.0–10.0 mM. Germinating CG spores were relatively more sensitive to SA than were spores of MF or PESP. The consistent nature of the dose-dependent inhibition of fungal growth in SA-amended PDA is shown by Table 2, which summarizes data from three consecutive experiments with BC.

Synergistic Inhibition of BC by SA and the Pro-oxidant Herbicide, PQ

SA and PQ were found to inhibit growth of BC on PDA in a synergistic manner (Table 3, Figure 1). Growth of BC on PDA amended with combinations of 2.0 mM SA and 4.0 or 8.0 mM PQ was substantially less than that predicted by an additive model of interaction, and these interactions were judged to be synergistic in nature. (See Material and Methods section for details of the evaluation of interactions.) Synergistic inhibition of BC on PDA was also observed for other combinations of SA and PQ (5.0 mM SA with 4.0 or 8.0 mM PQ; data not presented). In a well-plate test

Table 2. Dose-dependent inhibition of *Botrytis cinerea* by sodium salicylate (SA) in potato-dextrose agar. Data presented are from three replicate experiments, and each value is the mean ± SE colony diameter (mm) of four replicate Petri plates.

Trial	0.0 mM SA	0.2 mM SA	0.5 mM SA	1.0 mM SA	2.0 mM SA	5.0 mM SA	10.0 mM SA
1	67.1 ± 0.7	65.9 ± 0.8	60.5 ± 1.4	52.4 ± 1.8	36.4 ± 1.5	24.4 ± 1.8	12.4 ± 1.7
2	66.5 ± 0.7	63.3 ± 0.6	57.5 ± 0.5	50.0 ± 0.4	34.4 ± 1.3	25.6 ± 1.4	19.5 ± 2.3
3	67.5 ± 2.5	66.0 ± 1.1	62.3 ± 1.7	56.4 ± 1.6	36.8 ± 1.8	27.3 ± 2.2	15.6 ± 3.6

of the interactive effects of SA and PQ on BC, the fungus grew to fill all six wells per treatment that had been amended individually with 0.0 or 16.0 mM PQ or 2.0 or 5.0 mM SA, but did not grow in wells amended with 16.0 mM PQ and 2.0 or 5.0 mM SA (data not shown).

Synergism of SA with Cupric Chloride

Results of two replicate trials of an experiment designed to evaluate the potential interactive effects of SA and Cu on BC mycelial growth in well-plate culture are presented in Table 4. SA alone caused only a slight reduction in mycelial growth of BC, whereas growth of BC decreased gradually with increasing concentrations of Cu alone, and complete inhibition of BC growth was observed only with 10.0 mM Cu. In contrast, complete inhibition of BC growth occurred with 5.0 mM Cu (first experiment) or 2.0 mM Cu (second experiment) in the presence of 2.0 mM SA. Data regarding the synergistic inhibition of CG spore germination by SA and Cu are presented in Table 5. SA alone, at concentrations of 0.2 and 0.5 mM, did not affect the frequency of spore germination, whereas 0.75 mM SA inhibited mean germination by 61%, relative to controls. No germination of CG spores was observed in the presence of 1.0 or 2.0 mM SA (data not presented). In the absence of SA, CG spore germination was progressively inhibited by increasing Cu concentrations. Strong synergistic interactions were observed in combinations of 0.75 mM SA with Cu. For example, although percent spore germination relative to controls (OPC) was 94 and 39 for 0.2 mM Cu and 0.75 mM SA, respectively, this combination resulted in an OPC of 4% spore germination, much lower than the 37% germination predicted by the additive model. Interestingly, 0.2 and 0.5 mM SA effectively abolished the inhibition of spore germination by 0.2, 0.35, and 0.5 mM Cu, and SA thus appeared to act as an antagonist of Cu fungitoxicity toward CG spores in these combinations.

In a preliminary experiment with PESP, the combination of 2.0 mM SA with 0.5 mM Cu completely prevented spore germination, whereas these concentrations of SA or Cu alone appeared to have little or no effect on the frequency of spore germination (data not shown). In contrast to results obtained with CG, the presence of 0.2 or 0.5 mM SA did not alleviate the moderate inhibition of PESP germ tube elongation occasioned by 0.2 or 0.5 mM Cu (data not shown).

Table 3. Synergistic inhibition of *Botrytis cinerea* mycelial growth in potato-dextrose agar amended with sodium salicylate (SA) and/or paraquat (PQ). Data presented are mean colony diameters (mm) ± standard error for three replicate plates per treatment combination. PQ was mixed into partially cooled molten agar prior to pouring into Petri plates (20 ml/plate), whereas SA was introduced as 0.2 ml of a concentrated stock (100×) to the centers of plates and permitted to diffuse evenly throughout agar (as assessed by SA fluorescence under ultraviolet illumination). Other abbreviations: OPC, observed percent of control; AMI, additive model of interaction; PPC, predicted percent of control; SYN, synergistic interaction.

	PQ 0.0 mM	PQ 4.0 mM	PQ 8.0 mM
SA 0.0 mm	83.3 ± 0.4	25.7 ± 2.5	13.8 ± 3.2
OPC	100	31	17
SA 2.0 mM	67.2 ± 1.9	10.0 ± 1.6	4.2 ± 0.1
AMI	—	(80)(31)	(80)(17)
PPC	—	25	14
OPC	80	12	5
	—	SYN	SYN

Synergistic Inhibition of BC and PESP by SA in Combination with Culture Fluids of Antagonistic Bacteria

The antifungal activities of BRCF and WCF toward BC and PESP were synergistically enhanced by 2.0 mM SA (Table 6; Figure 2). BC growth was not prevented by exposure to SA

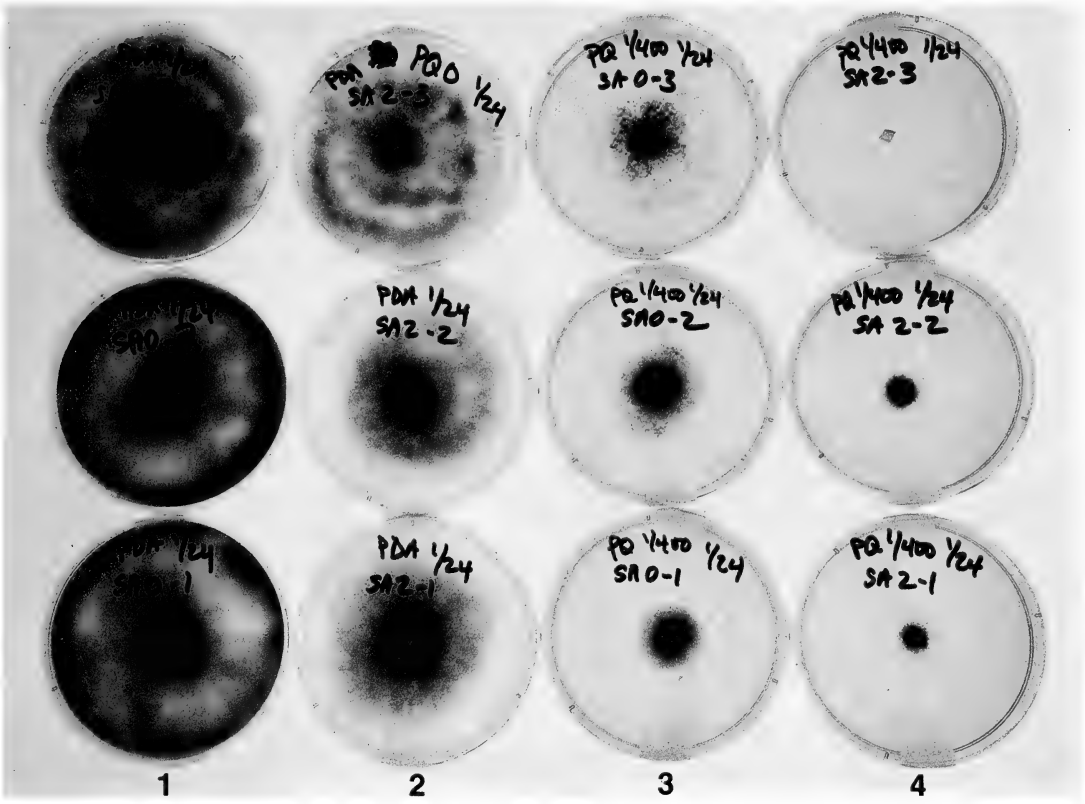

Figure 1. Synergistic inhibition of *Botrytis cinerea* (BC) mycelial growth in potato-dextrose agar PDA amended with sodium salicylate (SA) and/or paraquat (PQ). Pictured are all three replicate plates from the SA 2.0 mM-PQ 4.0 mM (1/400×) subset of a larger experiment whose data are presented in Table 3. The leftmost column of Petri plates (column 1) shows growth of BC in the absence of SA and PQ. Column 2 shows growth of BC in the presence of 2.0 mM SA. Column 3 shows growth of BC in the presence of 4.0 mM PQ. Column 4 shows growth of BC in the combined presence of 2.0 mM SA and 4.0 mM PQ.

Table 4. Synergistic inhibition of *Botrytis cinerea* mycelial growth in a liquid glucose-salts medium amended with sodium salicylate (SA) and cupric chloride (Cu). Data are of two replicate trials in which each SA-Cu treatment combination was represented twice in each of three replicate 24-well culture plates (n = 6 for each treatment combination). Values presented are mean ± standard error extent of radial mycelial growth (mm) from inoculum plugs, measured at 72 hours or 48 hours after BC inoculation (Trials I and II, respectively). Other abbreviations: OPC, observed percent of control; SYN, synergistic interaction.

	Cu 0.0 mM	Cu 0.5 mM	Cu 1.0 mM	Cu 2.0 mM	Cu 5.0 mM	Cu 10.0 mM
Trial I						
SA 0.0 mM	5.3 ± 0.2	4.5 ± 0.1	3.7 ± 0.1	2.0 ± 0.0	1.8 ± 0.1	0.0 ± 0.0
OPC	100	85	70	38	34	0
SA 2.0 mM	4.6 ± 0.1	3.5 ± 0.1	2.3 ± 0.1	0.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
OPC	87	66	43	13	0	0
	—	SYN	SYN	SYN	SYN	—
Trial II						
SA 0.0 mM	4.5 ± 0.1	3.5 ± 0.1	2.8 ± 0.1	2.0 ± 0.0	1.7 ± 0.1	0.0 ± 0.0
OPC	100	78	62	44	38	0
SA 2.0 mM	4.3 ± 0.1	2.7 ± 0.1	1.3 ± 0.1	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
OPC	96	60	25	0	0	0
	—	SYN	SYN	SYN	SYN	—

Table 5. Synergistic inhibition of *Colletotrichum graminicola* spore germination in a liquid glucose-salts medium amended with sodium salicylate (SA) and cupric chloride (Cu). Germination was assessed microscopically based on the presence (germinated) or absence (ungerminated) of an appresorium. No germ tubes were observed in the absence of appresoria. All spores in one microscopic field at 100× total magnification (>120 spores per well) were evaluated. Values presented are mean ± standard error for two replicate wells per treatment (n = 2). Other abbreviations: OPC, observed percent of control; ADD, ANT, and SYN denote additive, antagonistic, and synergistic interactions, respectively. No germination was observed in the presence of 1.0 or 2.0 mM SA, and these data are not presented in the body of the table.

	Cu 0.0 mM	Cu 0.2 mM	Cu 0.35 mM	Cu 0.5 mM
SA 0.0 mM	91.5 ± 1.1	86.0 ± 2.4	70.0 ± 2.1	37.5 ± 2.5
OPC	100	94	77	41
SA 0.2 mM	89.0 ± 0.0	84.0 ± 2.8	84.0 ± 2.8	86.5 ± 1.1
OPC	97	92	92	95
	—	ADD	ANT	ANT
SA 0.5 mM	96.5 ± 0.4	96.0 ± 0.0	91.5 ± 1.8	89.5 ± 0.4
OPC	105	105	100	98
	—	ANT	ANT	ANT
SA 0.75 mM	36.0 ± 7.1	4.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0
OPC	39	4	1	0
	—	SYN	SYN	SYN

or BRCF alone. However, the combination of SA with 100 or 200 µl of BRCF prevented BC growth in 2/3 and 3/3 wells, respectively. Neither BRCF nor SA alone prevented PESP growth at the doses employed. However, the addition of SA prevented PESP growth in 2/3 wells that received 50 µl of BRCF, and in all three wells that received 100 or 200 µl of BRCF. Only a slight effect of SA on inhibition of BC by WCF was observed, whereas growth of PESP was uniformly prevented in all combinations of SA and WCF.

The Interactive Effects of SA and NM

These effects varied with NM source [NM concentrate (NMC) or ready-to-use (NMRTU) formulations], SA and NM doses employed, timing of fungal inoculation following addition of NMRTU to GS, and species of test fungus. In trials with NMC, little or no inhibition of BC mycelial growth was observed with NMC alone or in combination with SA, although a transient delay in pigmentation was commonly observed in NMC-SA combinations (data not presented). In an experiment in which NMRTU was added to wells immediately prior to inoculation with BC, only a slight inhibition of BC by the highest dose of NMRTU was observed in the absence of SA (Table 7). Amendment with 2.0 mM SA only slightly enhanced inhibition by NMRTU dilutions of 1/10× and 1/5× (data not shown). However,

Table 6. Synergistic inhibition of mycelial growth of *Botrytis cinerea* (BC) and *Pestalotia* sp. (PESP) in a liquid glucose-salts medium amended with sodium salicylate (SA) and bacterial culture fluids (BCF). Single 24-well culture plates were filled with 0.5 ml GS per well to which were added 0, 50, 100, or 200 µl of BCF from the 18 d potato-dextrose broth shake cultures of an unidentified mixed white bacterial culture (WCF) or an unidentified mixed brown culture (BRCF). Fungal growth was assessed as present or absent for each of three replicate wells per treatment (one 24-well culture plate per BCF-fungus combination).

	WCF (µl/well)				BRCF (µl/well)			
	0	50	100	200	0	50	100	200
Incidence of BC mycelial growth								
SA 0.0 mM	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3
SA 2.0 mM	3/3	2/3	3/3	1/3	3/3	3/3	1/3	0/3
Incidence of PESP mycelial growth								
SA 0.0 mM	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3
SA 2.0 mM	3/3	0/3	0/3	0/3	3/3	1/3	0/3	0/3

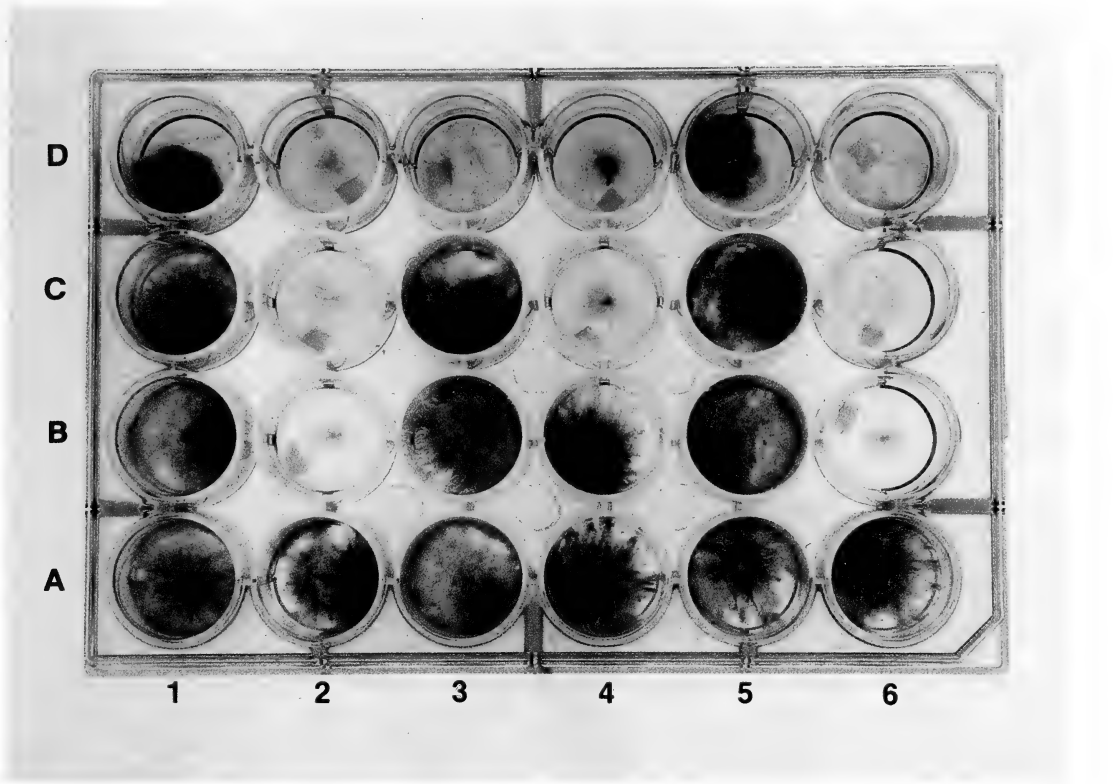

Figure 2. Synergistic inhibition of *Pestalotia* sp. (PESP) in a liquid glucose-salts medium amended with sodium salicylate (SA) and/or fluids from the 18 day potato-dextrose broth culture of an unidentified mixed brown bacterial culture (BRCF). Columns 1, 3, and 5 contained no SA, whereas columns 2, 4, and 6 were amended with 2.0 mM SA (final concentration). Rows A, B, C, and D were amended with 0, 50, 100, 200 μl of BRCF, respectively. Plating of BRCF aliquots onto potato-dextrose agar revealed the absence of live bacteria (no growth).

a strong synergism was observed in combinations of 4.0 mM SA with the higher NMRTU concentrations employed (1/20×, 1/10×, and 1/5× dilutions). Microscopic investigations revealed that diverse bacteria were detectable in both NMC and NMRTU products (when plated onto PDA), but only those in NMRTU appeared to multiply after addition to GS. Because a review of several preliminary experiments revealed that inhibition of fungi by

Table 7. Synergistic inhibition of *Botrytis cinerea* mycelial growth by sodium salicylate (SA) and a ready-to-use commercial formulation of neem oil extract (NMRTU). NMRTU dilutions were achieved by adding 0–100 μl NMRTU to wells containing sufficient liquid glucose-salts medium to yield 0.5 ml total volumes. Final SA concentrations of 4.0 mM were achieved by adding 20 μl of a 100 mM SA stock per well. The extent of mycelial growth (mm) from inoculum plugs was measured after a 48 hour incubation period. Data are mean ± standard error of six replicate wells (n = 6), for each SA 0.0 mM-NMRTU combination, and three replicate wells (n = 3) for each SA 4.0 mM-NMRTU combination. Other abbreviations: OPC, observed percent of control.

	NMRTU 0	NMRTU 1/100×	NMRTU 1/50×	NMRTU 1/20×	NMRTU 1/10×	NMRTU 1/5×
SA 0.0 mM	4.3 ± 0.1	4.2 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.0 ± 0.2
OPC	100	98	88	86	86	70
SA 4.0 mM	3.7 ± 0.2	3.3 ± 0.2	2.7 ± 0.2	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
OPC	(86)	77	63	16	0	0
	—	SYN	SYN	SYN	SYN	SYN

Table 8. Effect of timing of addition of sodium salicylate (SA) and a commercial ready-to-use formulation of a neem oil extract (NMRTU), relative to inoculation with *Botrytis cinerea,* on synergistic inhibition of mycelial growth. Data presented are growth incidence (GI, presence or absence) in three replicate wells per treatment combination (n = 3); and growth rate (GR) as mean ± standard error for growth relative to controls, with a rating of 0 indicating no growth; 1,≤10%; 2, 10–50%; 3, 51–75%; 4, 75–90%; or, 5, 91–100% growth relative to controls. No pretreatment indicates that NMRTU and SA were added to wells on the same day as BC inoculation, whereas 3-day pretreatment indicates that NMRTU and SA were introduced 3 days before BC inoculation. Other abbreviations: OPC, observed percent of control; ADD, ANT, and SYN denote additive, antagonistic, and synergistic interactions, respectively.

	NMRTU dilutions							
	0		1/100×		1/20×		1/5×	
	GI	GR	GI	GR	GI	GR	GI	GR
No Pretreatment								
SA 0.0 mM	3/3	5.0 ± 0.0	3/3	5.0 ± 0.0	3/3	4.0 ± 0.0	3/3	2.3 ± 0.1
OPC		100		100		80		46
SA 2.0 mM	3/3	4.0 ± 0.0	3/3	4.0 ± 0.0	3/3	4.0 ± 0.0	3/3	2.0 ± 0.0
OPC		80		80		80		40
		—		ADD		ANT		ANT
Three Day Pretreatment								
SA 0.0 mM	3/3	5.0 ± 0.0	3/3	4.0 ± 0.0	3/3	3.0 ± 0.0	3/3	3.0 ± 0.0
OPC		100		80		60		60
SA 2.0 mM	3/3	5.0 ± 0.0	1/3	0.3 ± 0.1	1/3	0.3 ± 0.1	0/3	0.0 ± 0.0
OPC		100		0.1		0.1		0
		—		SYN		SYN		SYN

combinations of NMRTU and SA was most consistent, frequent, and extensive when inoculation with BC had occasionally been delayed by one or more days after amendment of wells with NMRTU and SA, the effect of varied duration (0, 1, 2, or 3 days) of such delay was formally investigated. Data regarding the effects of a 0 or 3 d delay are presented in Table 8. Plates that received NMRTU, SA, and BC on the same day exhibited mycelial growth in all wells of each treatment combination, and interactions of SA and NMRTU were found to be either additive or slightly antagonistic. In contrast, when BC inoculation was delayed until 3 days after addition of SA and NMRTU, synergistic inhibition of BC growth was observed in all SA-NMRTU treatment combinations. Turbidity associated with bacterial growth was much greater in plates that received NMRTU (with or without SA) 3 days prior to inoculation with BC than in wells that received NMRTU and SA immediately prior to BC inoculation. These differences in turbidity were observed both at the time of inoculation with BC and at 3 days post-inoculation, when BC growth was assessed. Interestingly, the addition of SA and NMRTU 1 day before BC inoculation resulted in only slight development of turbidity and no inhibition of BC, whereas turbidity and extent of BC inhibition were of intermediate degree when BC inoculation occurred 2 days after addition of SA and NMRTU to wells (data not shown). The interactive effects of NMRTU and SA on mycelial growth of PESP were also examined. Again, little impact of NMRTU (alone or in combinations with SA) was observed when NMRTU and SA amendment occurred on the same day as BC inoculation (data not presented). In an experiment in which NMRTU and SA addition preceded BC inoculation by 2 days (Table 9), NMRTU alone reduced mean mycelial growth of PESP relative to controls in a dose-dependent manner, and the addition of SA resulted in a modest synergistic enhancement of fungal inhibition with most of the NMRTU doses employed.

DISCUSSION

Millimolar concentrations of SA were found to inhibit the growth and development of seven plant-pathogenic fungi in vitro, suggesting that sensitivity of fungal plant pathogens to mM SA concentrations may be a general phenomenon. Thus, SA concentrations equivalent

Table 9. Synergistic inhibition of mycelial growth of *Pestalotia* sp. by sodium salicylate (SA) and a ready-to-use commercial formulation of a neem oil extract (NMRTU). NMRTU dilutions were achieved by adding 0–100 μl NMRTU to wells containing sufficient liquid glucose-salts medium (GS) to yield 0.5 ml total volumes. SA concentrations were achieved by adding 10 or 20 μl of a 100 mM stock to yield final concentrations of 2.0 mM or 4.0 mM. The extent of mycelial growth (mm) from inoculum plugs was measured after a 48 hour incubation period. Data are mean ± standard error of four (SA 0.0 mM) or two (SA 2.0 mM) replicate wells per treatment. No PESP growth occurred in the presence of 4.0 mM SA (n = 2 per combination with NMRTU), and these data are omitted from the body of the table. Other abbreviations: OPC, observed percent of control; ADD and SYN denote additive and synergistic interactions, respectively.

	NMRTU 0	NMRTU 1/100×	NMRTU 1/50×	NMRTU 1/20 ×	NMRTU 1/10×	NMRTU 1/5×
SA 0.0 mM	6.0 ± 0.2	4.0 ± 0.0	3.5 ± 0.1	2.8 ± 0.1	1.9 ± 0.1	0.9 ± 0.1
OPC	100	67	58	47	32	15
SA 2.0 mM	4.5 ± 0.4	3.0 ± 0.0	2.0 ± 0.0	1.5 ± 0.4	1.0 ± 0.4	0.0 ± 0.0
OPC	75	50	33	25	17	0
	—	ADD	SYN	SYN	SYN	SYN

to those commonly used for the experimental induction of disease resistance in plants (2.0–10.0 mM) are potentially directly antifungal as well. Sensitivity of plant-pathogenic fungi to SA is consistent with prior published reports of the inhibition of saprophytic fungi by SA (Cruess and Irish 1931). However, concentrations of SA equivalent to those reported to occur in infected plant tissues (10.0–100.0 μM endogenous SA) did not inhibit the growth of BC in vitro. Thus, it seems unlikely that endogenous levels of SA in infected plant tissues (10–100 μM) would alone be capable of inhibiting growth of pathogens in plants, as proposed by Ruffer et al. (1995). This does not rule out the possibility that SA may act in concert with other substances produced by plants to limit pathogen activity (see below).

We observed multiple instances of in vitro synergism of SA with diverse other antifungal materials (PQ, Cu, BCF, and NMRTU). The mechanisms underlying these synergistic interactions remain to be determined. BC produces a wide variety of enzymes that degrade reactive oxygen species (Steel and Nair 1993; Choi et al. 1997; Gil-ad et al. 2000) that might be inhibited by SA as are some related plant enzymes (Chen et al. 1993; Durner and Klessig 1995; Slaymaker et al. 2002). Another plausible mechanism for the observed effects of SA is the suppression by SA of mitochondrial generation of ATP (Norman et al. 2004), which supplies energy necessary for many aspects of cellular life, including the production, operation, and maintenance of various detoxification mechanisms. In the case of Cu, SA may also increase entry of the toxicant into fungal cells via chelation. However, this would not likely account for the observed antagonistic interactions of lower concentrations of SA with Cu seen with CG but not with PESP. In the case of NMRTU, matters seem more complex, and may involve multiple interactions of NMRTU active or inert ingredients, contaminating bacteria, and SA. It is also plausible that acidification of the growth medium by antifungal materials or by fungi in response to same (not examined in the present studies) may result in increased uptake of salicylate, which is more lipid-soluble and more fungitoxic in the protonated form (Cruess and Irish 1931). Whether SA may usefully synergize the antifungal activities of commercial synthetic fungicides also remains to be determined.

We speculate that SA may be of practical utility as a multi-functional component (inducer of local disease resistance, direct antifungal agent, modifier of the composition and size of microfloral populations on plant surfaces, and synergist of other antifungal materials) of new plant disease-control formulations that combine ingredients of synthetic chemical and/or biological origin. Such combinations may delay or prevent development of pathogen resistance to conventional fungicides, and may also enable reduced application rates for these fungicides (Ye et al. 1995). For example, fixed copper fungicides such as Bordeaux mixture are often applied at rates that deliver approximately 1–2 kg of elemental copper per acre per application, sometimes resulting in the accumulation of copper to phytotoxic levels in

soils. Enhancement of the fungitoxicity of cupric ions by SA may permit large reductions in the total amount of Cu applied for plant disease management, thereby reducing or eliminating problems from Cu accumulation. Evaluation of these possibilities requires further testing of the effects of SA and other materials on the outcomes of plant-pathogen interactions in controlled and field environments.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of two mini-grants from the Bluegrass Community and Technical College, 470 Cooper Drive, Lexington, KY 40506-0235, without which our research would not have been possible. We also give thanks to Dr. William Witt, Department of Agronomy, University of Kentucky, Lexington, KY 40506, for his gift of Gramoxone (PQ), and to Dr. Lisa Vaillancourt, Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40506, who provided us with a culture of *Colletotrichum graminicola* (CG).

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Kentucky Baccalaureate Origins of Doctorate Recipients in the Biological Sciences, Chemistry, and Physics, 1978 through 2002

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ABSTRACT

This paper presents the Kentucky baccalaureate origins of men, women, and under-represented group U.S. citizen doctoral recipients in the biological sciences, chemistry, and physics from 1978 through 2002. Counterintuitive results are reported for chemistry and physics. For these disciplines, institutions with an undergraduate focus and smaller science infrastructure have outperformed Kentucky's research universities.

Further, the results suggest that little or no attention has been paid in a systemic manner to any Kentucky under-represented minority group during this period. Conversely, there has been significant growth in the percentage and absolute numbers of U.S. women earning doctoral degrees in the biological sciences and in chemistry after receiving Kentucky baccalaureate degrees.

INTRODUCTION

Higher education graduate and undergraduate programs play a pivotal role in developing the intellectual capital of the scientific and technical workforce. This paper presents the Kentucky baccalaureate origins of men, women, and underrepresented group doctoral recipients in the biological sciences, chemistry, and physics from 1978 through 2002. It builds upon similar efforts in chemistry by Hall (1985) by using an expanded dataset that includes underrepresented groups and the additional disciplines of the biological sciences and physics. Results for the biological sciences, chemistry, and physics were targeted since these academic disciplines represent core competencies for any undergraduate program in the physical sciences.

The importance of undergraduate training in the sciences in production of a technological infrastructure and as a prelude to graduate study is supported by all Carnegie research-extensive and research-intensive universities as well as by the worldwide scientific community. For example, Dr. Frank Rhodes, former president of Cornell University, stated, "while research and teaching both contribute to the strength and vitality of the U.S. research university, it is undergraduate teaching and learning, that is the central task. Undergraduate education is fundamental to the existence of the university. . ." (Rhodes 1994).

Kentucky's transformation to a competitive, high-tech economy requires the development and enhancement of the scientific and technological workforce. The critical nature of this effort is reflected in numerous state-supported and federally partnered initiatives such as the Experimental Program to Stimulate Competitive Research (NSF-EPSCoR Program 2005), Institutional Development Award (NIH-IDeA Program 2005), the Small Business Innovation Research/Small Business Technology Transfer (NSF-SBIR/STTR Program 2005). In recent years, comprehensive statewide efforts to improve higher education include significant budget reallocations through the Research Challenge Trust Fund initiative (STTR-EPSCoR Program 2005). The vast majority of these funds are directed toward improving the scientific and technological resources of Kentucky-based institutions of higher education. The basis for such efforts appears driven by research competitiveness within higher education (Teich 1996) and the need to update and enhance the Kentucky technical, scientific, and educational infrastructure. The research or discovery undertakings at universities and colleges can lead to patents and commercial development. Therefore, technol-

ogy transfer, intellectual property, and royalty income are among the important issues directly linking the development of intellectual capital to the state's economic growth and viability.

METHODS

The Kentucky baccalaureate origins of doctorate recipients in the biological sciences, chemistry, and physics are derived from data provided by the Survey of Earned Doctorates (SED) and entered into WebCASPAR (Computer-Aided Science Policy Analysis and Research). The SED is conducted annually by the University of Chicago National Opinion Research Center for the Federal sponsors of the survey: the National Science Foundation (NSF), the National Institutes of Health (NIH), the U.S. Department of Education (USED), the National Endowment for the Humanities (NEH), the U.S. Department of Agriculture (USDA), and the National Aeronautic and Space Administration (NASA). The SED does not report professional degrees such as the D.D.S., D.V.M., J.D, M.D., and O.D. Information from this survey becomes part of the Doctorate Records File (DRF) and contains data on all earned doctorates granted by regionally accredited U.S. universities in all fields from 1920 to the present.

WebCASPAR is a database system (NSF-WebCASPAR 2005) available on the World Wide Web and containing information about academic science and engineering resources. Information from academic surveys of the Division of Science Resources Statistics (SRS) of the National Science Foundation and information from a variety of other sources, including but not limited to the SED, are included in the database. WebCASPAR currently can provide critical demographic data through access to most DRF doctoral records in the biological sciences, chemistry, and physics from 1966 through 2003.

Data for the SED are collected directly from individual doctorate recipients. The questionnaire is distributed through the cooperation of graduate deans to those who are completing their doctorates. The data for a given year include all doctorates awarded in the 12-month period ending on 30 June. Over the past decade, the self-report response rates to the SED questionnaire varied from a high of 94.7% in 1993 to a low of 91.2% in 2002. The response rate for a specific year may vary slightly over time as late responses are added. To minimize the effect of late responses, we used 2002 as the last year for our data survey.

Since, ''differences in survey and item response rates from year to year can produce numerical fluctuations that are unrelated to true trends'' (NSF Science Resources Statistics 2005), we used a 25-year time line (1978–2002) to minimize the effect of such fluctuations. Through this paradigm we addressed the stated concern that ''increasing or decreasing numbers in a citizenship or racial/ethnic group ... reflect to some degree any upward or downward change in both overall survey response and item response'' (NSF Science Resources Statistics 2005).

Until 1990, SED records with missing gender were imputed using the first name of the recipient only if the first name could be positively identified as male or female. Records that could not be identified by first name were set to male. In 1990, follow-up procedures were implemented for cases still missing after imputation. Records for which gender still could not be identified in the follow-up were set to male. In 1997, it was decided to discontinue this practice. Therefore, data with unknown gender occur in 1988 and in 1990 and later years. For each year beginning in 1990, however, the response rate is greater than 99.2% (Hill, S. T. 2005. Director, Doctorate Data Project, NSF Division of Science Resources Statistics, pers. comm., 11 Feb.).

As a result of new follow-up procedures implemented during the 1990 survey cycle, there was greater adjustment to the numbers for race/ethnicity and citizenship than in earlier years. Although these new procedures have improved the completeness of the data, they also created a break in trend data that should be taken into consideration when analyzing sensitive data such as race/ethnicity after 1989. The effect on the trend line for citizenship is extremely small, almost negligible. The effect on the trend line for race is minimal. To the extent that there has been an effect, blacks tend to be slightly overrepresented in institution-completed responses, so blacks would trend toward a larger proportion after the new procedures were implemented (Hill, S.T. 2005. Director, Doctorate Data Proj-

Table 1. Kentucky baccalaureate origins of U.S. citizen doctorate recipients in the biological sciences (1978–2002).

KY Baccalaureate Institution for Biology Doctorates	Totals	KY Rank	National Rank/1434	Totals Men	KY Rank	National Rank/1284	Totals Women	KY Rank	National Rank/1288
University of Kentucky	161	1	120	105	1	114	56	1	132
University of Louisville	84	2	223	50	2	228	34	2	214
Centre College	54	3	324	25	6	392	29	3	243
Western Kentucky University	52	4	333	29	5	346	23	4	301
Murray State University	48	5	351	35	3	306	13	5	428
Thomas More College	41	6	385	33	4	319	8	11	596
Eastern Kentucky University	36	7	421	24	7	409	12	6	455
Bellarmine College	28	8	488	17	8	492	11	7	483
Northern Kentucky University	25	9	523	16	9	513	9	8	558
Berea College	20	10	588	11	11	608	9	8	558
Georgetown College	20	10	588	11	11	608	9	8	558
Transylvania University	18	12	628	11	11	608	7	12	634
Morehead State University	17	13	649	12	10	585	5	14	735
Asbury College	14	14	716	7	14	719	7	12	634
Kentucky Wesleyan College	6	15	953	4	15	843	2	15	951
Kentucky State University	4	16	1043	3	16	904	1	17	1064
Union College	3	17	1101	3	16	904	na	na	na
Brescia College	2	18	1161	2	18	972	0	20	1246
Cumberland College	2	18	1161	0	20	1255	2	15	951
Campbellsville University	2	18	1161	1	19	1056	1	17	1064
Spalding University	1	21	1230	0	20	1255	1	17	1064
Total Academic Instiution	638			399			239		

ect, NSF Division of Science Resources Statistics, pers. comm. 11 Feb.).

RESULTS

Table 1 presents the Kentucky baccalaureate origins of doctoral recipients in the biological sciences for men and women from 1978 through 2002. A total of 638 baccalaureate degrees were awarded to individuals who received a doctorate in the biological sciences with 399 (63%) going to men and 239 (37%) going to women. Columns two, three, and four, respectively, present the total number of doctorates awarded, the rank among Kentucky institutions of higher education, and the national rank out of 1434 institutions in the SED database. Columns five, six, and seven, respectively, present the number of doctorates awarded to men, the rank among Kentucky institutions of higher education, and the national rank out of 1284 institutions in the SED database. Columns eight, nine, and ten, respectively, present the number of doctorates awarded to women, the rank among Kentucky institutions of higher education, and the national rank out of 1288 institutions in the SED database. Data entries of "na" indicate information not available or not entered in the SED database.

Table 2 presents the Kentucky baccalaureate origins of doctoral recipients in chemistry for men and women from 1978 through 2002. A total of 325 baccalaureate degrees were awarded to individuals who earned a doctorate in chemistry with 243 (75%) going to men and 82 (25%) going to women. Columns two, three, and four, respectively, present the total number of doctorates awarded, the rank among Kentucky institutions of higher education, and the national rank out of 1215 institutions in the SED database. Columns five, six, and seven, respectively, present the number of doctorates awarded to men, the rank among Kentucky institutions of higher education, and the national rank out of 1215 institutions in the SED database. Columns eight, nine, and ten, respectively, present the number of doctorates awarded to women, the rank among Kentucky institutions of higher education, and the national rank out of 967 institutions in the SED database. Data entries of "na" indicate information not available or not entered in the SED database.

Table 3 presents the Kentucky baccalaureate origins of doctoral recipients in physics for men and women from 1978 through 2002. A total of 107 baccalaureate degrees were awarded to individuals who received a doctorate in physics, with 98 (92%) going to men and 9 (8%) going to women. Columns two, three, and four, respectively, present the total number of doctorates awarded, the rank

Table 2. Kentucky baccalaureate origins of U.S. citizen doctorate recipients in chemistry (1978–2002).

KY Baccalaureate Institution for Chemistry Doctorates	Totals	KY Rank	National Rank/1215	Totals Men	KY Rank	National Rank/1215	Totals Women	KY Rank	National Rank/967
Western Kentucky University	48	1	185	37	1	185	11	2	178
University of Kentucky	46	2	191	31	2	231	15	1	120
University of Louisville	35	3	265	25	3	275	10	3	212
Murray State University	26	4	345	20	4	343	6	5	332
Centre College	25	5	356	19	5	357	6	5	332
Transylvania University	22	6	394	15	6	419	7	4	301
Berea College	18	7	449	14	7	446	4	8	442
Northern Kentucky University	17	8	466	13	8	472	4	8	442
Bellarmine College	13	9	548	11	10	515	2	11	627
Eastern Kentucky University	13	9	548	12	9	491	1	14	746
Kentucky Wesleyan College	12	11	567	8	11	587	4	8	442
Thomas More College	9	12	647	4	15	741	5	7	376
Morehead State University	9	12	647	7	12	615	2	11	627
Cumberland College	9	12	647	7	12	615	2	11	627
Georgetown College	8	15	677	7	12	615	1	14	746
Asbury College	3	16	887	3	16	789	na	na	na
Union College	3	17	887	2	17	855	1	14	746
Campbellsville University	2	18	960	2	17	855	na	na	na
Brescia College	2	18	960	2	17	855	na	na	na
Kentucky State University	2	18	960	2	17	855	na	na	na
Pikeville College	2	18	960	2	17	855	na	na	na
Spalding University	1	22	1054	na	na	na	1	14	746
Total Academic Institution	325			243			82		

among Kentucky institutions of higher education, and the national rank out of 955 institutions in the SED database. Columns five, six, and seven, respectively, present the number of doctorates awarded to men, the rank among Kentucky institutions of higher education, and the national rank out of 823 institutions in the SED database. Columns eight, nine, and ten, respectively, present the number of doctorates awarded to women, the rank among Kentucky institutions of higher education, and the national rank out of 474 institutions in the SED database. Data entries of "na" indicate information not available or not entered in the SED database.

Table 4 presents the Kentucky baccalaureate origins of doctoral recipients in the biological sciences from 1978 through 2002 for black men and black women. During that period, U.S. citizens in the biological sciences earned 81,951 doctorate degrees, of which 1538 (1.9%) went to black U.S. citizens. Ken-

Table 3. Kentucky baccalaureate origins of U.S. citizen doctorate recipients in physics (1978–2002).

KY Baccalaureate Institution for Physics Doctorates	Totals	KY Rank	National Rank/955	Totals Men	KY Rank	National Rank/823	Totals Women	KY Rank	National Rank/474
Thomas More College	20	1	176	19	1	167	1	3	227
Western Kentucky University	17	2	200	17	2	186	na	na	na
University of Kentucky	14	3	234	11	3	256	3	1	104
Eastern Kentucky University	13	4	252	10	4	278	3	1	104
Murray State University	11	5	285	10	4	278	1	3	227
University of Louisville	9	6	324	8	6	322	1	3	227
Berea College	7	7	374	7	7	353	na	na	na
Centre College	6	8	399	6	8	376	0	6	418
Bellarmine College	3	9	542	3	9	506	na	na	na
Northern Kentucky University	2	10	590	2	10	567	na	na	na
Transylvania University	2	10	590	2	10	567	na	na	na
Cumberland College	1	12	672	1	12	649	na	na	na
Kentucky Wesleyan College	1	12	672	1	12	649	na	na	na
Union College	1	12	672	1	12	649	na	na	na
Georgetown College	na	15	na	na	na	na	na	na	na
Morehead State University	na	15	na	na	na	na	na	na	na
Asbury College	na	15	na	na	na	na	na	na	na
Total Academic Institution	107			98			9		

Table 4. Kentucky baccalaureate origins of black U.S. citizen doctorate recipients in the biological sciences (1978–2002).

KY Baccalaureate Institution for Biology Doctorates Awarded to Black Students	Totals	KY Rank
University of Kentucky	3	1
Berea College	1	2
Kentucky State University	1	2
Murray State University	1	2
University of Louisville	1	2
Total Academic Institution	7	

tucky produced seven black baccalaureate recipients of doctoral degrees in the biological sciences.

Table 5 presents the Kentucky baccalaureate origins of doctoral recipients in the biological sciences from 1978 through 2002 for Hispanic men and Hispanic women. During that period U.S. citizens in the biological sciences earned 81,951 doctorate degrees, of which 1930 (2.4%) went to Hispanic U.S. citizens. Kentucky produced seven Hispanic baccalaureate recipients of doctoral degrees in the biological sciences.

Table 6 presents the Kentucky baccalaureate origins of doctoral recipients in chemistry from 1978 through 2002 for black men and black women. During that period, U. S. citizens earned 30,864 doctorate degrees in chemistry, with 553 (1.8%) of that number going to U.S. blacks. Kentucky produced four black baccalaureate recipients of doctoral degrees in chemistry.

Table 7 presents the Kentucky baccalaureate origins of doctoral recipients in chemistry from 1978 through 2002 for Hispanic men and Hispanic women. During that period U. S. citizens earned 30,864 doctorate degrees in chemistry, with 758 (2.5%) of that number going to U.S. Hispanics. Kentucky produced three Hispanic baccalaureate recipients of doctoral degrees in chemistry.

Table 5. Kentucky baccalaureate origins of Hispanic U.S. citizen doctorate recipients in the biological sciences (1978–2002).

KY Baccalaureate Institution for Biology Doctorates Awarded to Hispanic Students	Totals	KY Rank
University of Kentucky	3	1
Centre College	1	2
Murray State University	1	2
Thomas More College	1	2
University of Louisville	1	2
Total Academic Institution	7	

Table 6. Kentucky baccalaureate origins of black U.S. citizen doctorate recipients in chemistry (1978–2002).

KY Baccalaureate Institution for Chemistry Doctorates Awarded to Black Students	Totals	KY Rank
Berea College	1	1
Centre College	1	1
University of Kentucky	1	1
Western Kentucky University	1	1
Total Academic Institution	4	

Within Kentucky, institutions of higher education produced one black (from Centre College, 1979) and no Hispanic baccalaureate recipients who earned a doctorate in physics from 1978 through 2002. During that period U.S. citizens earned 15,938 doctorate degrees in physics. Of that number, black U. S. citizens earned 196 (1.2%) and Hispanic U.S. citizens earned 296 (1.9%).

Table 8 presents the average number of doctorates earned annually by U. S. citizens matriculating from baccalaureate institutions over two time periods: 1978 through 1994 (17 years) and 1995 through 2002 (8 years). Column one presents the discipline cohorts. Column 2 presents the average number of doctorates earned annually by individuals matriculating from baccalaureate institutions nationwide from 1978 through 1994 and from 1995 through 2002, as well as the percentage change between these averages. Column 3 presents similar data for individuals matriculating from Kentucky baccalaureate institutions.

DISCUSSION

Table 1 reports results for the production of doctorates whose baccalaureates are in the biological sciences. The University of Kentucky

Table 7. Kentucky baccalaureate origins of Hispanic U.S. citizen doctorate recipients in chemistry (1978–2002).

KY Baccalaureate Institution for Chemistry Doctorates Awarded to Hispanic Students	Totals	KY Rank
Centre College	1	1
Eastern Kentucky University	1	1
Western Kentucky University	1	1
Total Academic Institution	3	

Table 8. Comparison of national average annual doctorates with Kentucky baccalaureate-granting institutions average annual doctorates (1978–1994) and (1995–2002).

Discipline	National			Kentucky		
	⟨1978–1994⟩	⟨1995–2002⟩	%Change	⟨1978–1994⟩	⟨1995–2002⟩	%Change
Biological Sciences						
Overall	3148	3555	12.93%	23.3	30.3	30.04%
Women	1109	1670	50.59%	7.88	13.1	66.24%
Black	43.6	99.5	128.21%	0.24	0.38	58.33%
Hispanic	54.2	126	132.47%	0.18	0.5	177.78%
Chemistry						
Overall	1276	1148	−10.03%	12.5	14.125	13.00%
Women	263	355	34.98%	2.88	4.13	43.40%
Black	17	34	100.00%	0.12	0.25	108.33%
Hispanic	28	35	25.00%	0.18	0	−100.00%
Physics						
Overall	636	641	0.79%	4.47	3.88	−13.20%
Women	48.4	80.1	65.50%	0.29	0.50	72.41%
Black	6.35	11	73.23%	0	0.125	—
Hispanic	10.3	15.1	46.60%	0	0	0%

and the University of Louisville, as might be expected due to their size and available resources, produce the greatest number of baccalaureates who earn doctorates. Kentucky institutions appear to lag behind national benchmarks with the highest ranking (University of Kentucky) in the biological sciences being 120 out of 1434 schools in the SED database. Although this ranking is above many institutions with far fewer resources and significantly smaller student populations, it ranks significantly below benchmark institutions of comparable mission and student population. For example, it ranks significantly below the University of Georgia ranked at 58, the lowest of 20 benchmarks (UK Benchmarks 2005).

Achieving parity with leading benchmark institutions appears to require implementation of comprehensive institutional approaches to strengthen STEM teaching and learning. At a minimum, such institutional approaches must necessarily focus on support for faculty, appropriate instrumentation, and improvement of access, retention, and graduation rates for all students. Further analysis of benchmark programs relative to those in Kentucky should yield greater insight into the substantial observed differences.

Tables 2 and 3 report results that appear counterintuitive for the production of baccalaureates in chemistry and physics. Western Kentucky University and Thomas More College, institutions primarily devoted to undergraduate education with relatively limited resources and relatively smaller enrollments, have assumed leadership in the production of intellectual capital, respectively, in chemistry and physics. In physics, Thomas More College and Western Kentucky University together produced 60% more baccalaureates that earned doctorates than the University of Kentucky and the University of Louisville combined.

Given the robust nature of the science infrastructure at the University of Kentucky and at the University of Louisville, (e.g., seminars, information technology resources, research opportunities for undergraduate students, and targeted NSF and NIH scientific infrastructure support), the relatively low numbers of baccalaureates earning doctorates in chemistry and physics are surprising. Detailed analysis of undergraduate programs and institutional commitment offer promising areas for future study. Kentucky institutions appear to lag far behind national benchmarks, with the highest ranking in chemistry being 185 out of 1215 schools in the SED database. The highest ranking for a Kentucky research-extensive university is 191, significantly below the lowest of 20 benchmarks, the University of Iowa at 109.

Kentucky institutions appear to lag far behind national benchmarks, with the highest ranking in physics being 176 out of 955 schools in the SED database. The highest ranking for a Kentucky research-extensive

university is 234, above the lowest ranked benchmark, Georgia at 305, but significantly below the next lowest benchmark, University of Iowa at 59.

Tables 4 through 7 present results for the production of doctorates from underrepresented minority group candidates whose baccalaureates are in the biological sciences and chemistry. No results were tabulated for physics since only one doctorate was earned over the 25 years of collected data.

The results suggest that little or no attention was paid in a systemic manner to any Kentucky underrepresented minority group from 1978 through 2002. During that time, the percentage and absolute numbers of U.S. underrepresented minority group students receiving baccalaureate degrees and then earning doctorates in the biological sciences, chemistry, and physics roughly doubled, but little change was reported for such individuals in Kentucky.

Table 8 presents the annual averages of Kentucky baccalaureate origins of doctorate production from 1978 through 1994 (17 years) and from 1995 through 2002 (8 years). These time periods were chosen to reflect the impact of large-scale or systemic funding during the late 1980s and early 1990s by the NIH and the NSF. Of particular interest were the NSF-funded Statewide Systemic Initiative programs, including the Rural Systemic Initiative Program (targeting pre-college students) and the Alliances for Minority Participation Program (targeting college undergraduates). These programs were introduced nationwide and targeted to address perceived deficiencies in the growth of the scientific and technological workforce. The effects of these programs should have been evident as early as 1995 on the production of doctorates in sciences (Hicks, A. J. 2004. Program Director, NSF-LS AMP Program, Division of Human Resource Development, pers. comm., 11 Dec.). Thus, we chose to look at changes in doctorate degree production during these periods and compare them to national trends. Of the NSF systemic programs that would directly affect doctorate production from baccalaureate institutions, Kentucky universities participated only in the Rural Systemic Initiative, a program that focused on the needs of women and of the rural economically disadvantaged.

Our data with respect to women doctorates are consistent with those reported by Everett and DeLoach (1991) and more recently by Freeman et al.(2004). There has been significant growth in the percentage and absolute numbers of U.S. women receiving baccalaureate degrees and then earning doctorates in the biological sciences and chemistry both nationwide and in Kentucky. The rate of growth, using results solely from 2002 and 1978, seems more rapid than Table 8 would suggest. If 2002 is used as a measure, then in the biological sciences, 47.1% of U.S. doctorates (up from 25.1% in 1978) were awarded to women, while in Kentucky 51.4% (up from 20.8% in 1978) were awarded to women.

In chemistry a similar trend increase was observed with 34.3% of U.S. doctorates (up from 12.6% in 1978) awarded to women, while in Kentucky 27.3% (up from 0% in 1978) were awarded to women.

In physics, the overall number of doctorates awarded decreased nationwide, with women earning 4.8% of U.S. doctorates (down from 11.5% in 1978), while in Kentucky women earned 0% in 2002 and 0% in 1978.

The results with respect to underrepresented minorities suggest that most numerical analyses would be questionable since so very few individuals who earned a baccalaureate in Kentucky subsequently earned a doctoral degree in the sciences.

However, these results are consistent with (a) a highly successful NSF-sponsored and state-partnered Appalachian Rural Systemic Initiative program designed to increase the numbers of women and economically disadvantaged Kentuckians entering the scientific and technological workforce; and (b) the lack of any systemic program (e.g., NSF–Louis Stokes Alliances for Minority Participation, NIH-Minority Access to Research Careers) designed to increase the numbers of underrepresented minority Kentuckians entering the scientific and technological workforce.

The baccalaureate origins of doctoral recipients in core competencies (the biological sciences, chemistry, and physics) in the physical sciences provide reasonable benchmarks to test the quality of science, technology, engineering, and mathematics (STEM) baccalaureate programs.

SUMMARY

These findings support the conclusion that the Kentucky higher education system failed to enhance the production of black and Hispanic physical science majors who later earned doctorates. Further, these findings speak to a lost opportunity during the promising equal opportunity era (ca. 1970 through 1995) to engage, more fully, underrepresented minority groups in the scientific and technological workforce. Conversely, during this period significant progress was made to support and encourage female and economically disadvantaged representation in the intellectual leadership of the physical sciences.

These results suggest that constructive enhancements to the Kentucky science and technology policies should be made to address its deficiencies in order to realize the potential of the intellectual capital inherent in minority communities. The training and development of intellectual leadership in the physical sciences for women and the economically disadvantaged has already yielded significant benefits. Similar efforts in the black and Hispanic communities should help Kentucky realize the full extent of economic and quality of life advantages provided by an educated populace.

ACKNOWLEDGMENTS

Useful conversations with Dr. Luther Williams, the William T. Kemper Director of Education of the Missouri Botanical Garden, are acknowledged.

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J. Ky. Acad. Sci. 66(2):137–138. 2005.

NOTES

Host Occurrence of Eastern Mistletoe *(Phoradendron leucarpum*, Viscaceae) in Robertson County, Kentucky—A survey of eastern mistletoe (*Phoradendron leucarpum*)-infested host trees in Robertson County, Kentucky, was conducted from 27 Dec 2002 to 6 Jan 2003. All paved roads and passable gravel roads in the county were traveled by vehicle. Host trees were identified and tallied by species, and the number of clumps of mistletoe were recorded. Voucher specimens were obtained for each representative host tree species by a extendable 12 m fiberglass linesman pole and then mounted, labeled, and deposited in the Berea College Herbarium (BEREA).

Four mistletoe studies have been published in Kentucky (1, 2, 3, 4). The only published report for mistletoe in Robertson County had been a sight record on *Juglans nigra* by Reed and Reed (1).

Robertson County is located in northeastern Kentucky (Figure 1). With 259 km^2 of land area, it is the smallest Kentucky county. Mount Olivet, the county seat, is centrally located at latitude 38°31′55″ N and longitude 84°02′14″ W. Robertson County is the state's least populous county, having 2266 people; 287 people reside in Mount Olivet according to the 2000 census (5).

The county is located entirely in the Hills of the Bluegrass ecoregion of the Interior Plateau Region of Kentucky (6). The hills consist of upland rolling topography with moderately steep slopes and broad ridges. Small ravines form seasonally intermittent streams leading to larger streams, e.g., North Fork Licking River and Licking River. Elevations in the county range from 170 m to 308 m. In Robertson County, Upper Ordovician limestone and shale bedrock belong to the Clays Ferry Formation, Lexington Limestone, Kope Formation, and Fairview Formation (7). The Eden-Nicholson-Lowell soil association is found throughout the Robertson County upland terrain (8).

Braun (9) classified forest vegetation as Western Mesophytic Forest in this region of the eastern deciduous forest; Küchler (10) placed northeastern Kentucky in the *Quercus-Carya* forest. I observed forest vegetation on upland limestone and shale hills as *Quercus alba-Carya* spp. forest with scattered large stands of *Juniperus virginiana* and a riparian forest of *Acer negundo-Platanus occidentalis-Acer saccharinum* in alluvial areas. A majority of the county is composed of pasturelands and agricultural croplands.

The continental climate in northeastern Kentucky is characterized by cool to cold winters and warm, humid summers with precipitation well distributed throughout the year (11). Climate data, 1977–2000, are from the Falmouth weather station in Pendleton County, 32 km west of Robertson County (12). Mean annual precipitation is 111 cm with the lowest precipitation, 7.2 cm, in October and the highest, 11.8 cm, in May and July. Mean annual temperature is 11.7°C with the mean lowest temperature, −1.6°C, in January and the highest temperature, 23.8°C, in July. The mean growing season is 175 days with a range of 132 days to 211 days. The median first fall frost is 15 October and the last spring frost is 24 April (12).

Phoradendron leucarpum was found only on 45 trees from 7 host tree species in 6 families. *Juglans nigra* accounted for 23 host trees (51.1%). Other host trees were 10 *Ulmus americana* (22.2%), 6 *Robinia pseudoacacia* (13.4%), 3 *Acer saccharinum* (6.7%), and 1 each of *Gleditsia triacanthos, Fraxinus americana,* and *Prunus serotina* (6.6%). All host trees were situated east of Mount Olivet in the eastern half of the county (Figure 1). The sparse number of host trees in Robertson County was very significant when compared to other recent mistletoe studies in central and south central Kentucky, i.e., Lexington-Blue Grass Army Depot in Madison County with 1837 host trees (2), Rockcastle County with 3502 trees (3), and Garrard County with 1740 (4).

Low winter temperatures are the principal factor responsible for the sparse number of mistletoe-infested trees in Robertson County, e.g., in January 1994 the lowest temperature was −34.4°C and in February 1996 it was −22.1°C. My observations of host trees in mistletoe surveys showed extensive mistletoe die-back to the endophytic root system when extremely low temperatures have occurred. Spooner (13) found that eastern mistletoe reaches its northernmost distribution range in southern Ohio. He produced evidence that the main reason for the northern limits of eastern mistletoe corresponded to the mean minimum January temperature of −4.5°C. My extensive reconnaissance has shown that the incidence of mistletoe is sparse not only in Robertson County, but in all of the northern Kentucky counties within the Hills of the Bluegrass and Outer Bluegrass ecoregions. In several of these northern counties, I have observed eastern mistletoe only once or twice from one to three host tree species for the entire county, e.g., Bracken, Campbell, Lewis, Gallatin, Kenton, Owen, and Pendleton counties. Eastern mistletoe has not been observed in Grant County despite repeated searches.

Other mistletoe studies have documented the effects of low temperatures on spread and mortality of mistletoes. Garman (14) reported that severe winters destroyed much eastern mistletoe in Kentucky, but mistletoe gradually reappeared under more favorable temperatures in later years. Deam (15) observed the detrimental effects of low temperatures on eastern mistletoe and the restriction of the plant's Indiana range to the southern part of the state. Lightle (16) documented that low temperatures were factors in injury and mortality to three *Phoradendron* spp. in southern Arizona and New Mexico. Wagener (17) concluded that very low temperatures restricted two *Phoradendron* spp. to their present distribution limits in California and explained their absence in other parts of the hosts' range.

The dioecious characteristic of mistletoe may be another important factor in the overall sparse numbers of mis-

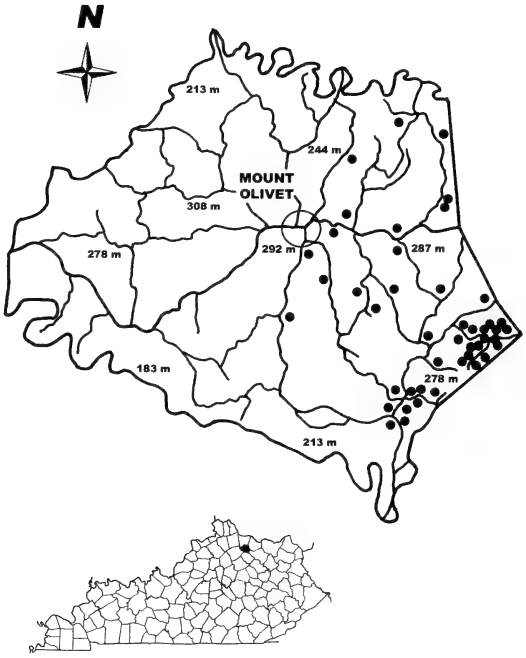

Figure 1. Robertson County, in northeastern Kentucky. The forty-five mistletoe-infested tree locations are indicated with solid circles.

tletoe-infested trees. The chance of mature fruits from a pistillate plant becoming dispersed by birds and established in new host trees with both sexes present in the near vicinity may be rare especially when there are so few mistletoe-infested trees in the county. Most the 45 host trees tended to be lightly infested; 19 trees had only a single clump, 7 trees had two clumps, and the remaining 19 trees had from 3 to 15 clumps. Thus, few trees had both staminate or pistillate plants in the same tree or nearby infested trees. Several host trees were isolated in upland pasturelands or around residences. Other mistletoe-infested trees of the same species along upland roads or fencerows tended to show an aggregated or clustered pattern (Figure 1).

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List of Recent Reviewers

We gratefully acknowledge the contribution of time and expertise provided by the following individuals in reviewing manuscripts submitted for consideration by the *Journal of the Kentucky Academy of Science.*

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J. Ky. Acad. Sci. 66(2):140–142. 2005.

Press Release:

Kentucky Academy of Sciences (KAS) Resolution In Support of Evolution

At the annual business meeting of the KAS, on November 12, 2005, the KAS reviewed and reaffirmed past resolutions in support of the teaching of Evolution and unanimously endorsed the American Association for the Advancement of Sciences's "Resolution on Intelligent Design Theory"

The following resolution, already adopted by the Kentucky Academy of Science at the annual business meeting on November 14, 1981, was unanimously approved again at its annual business meeting on November 12, 2005:

> The Kentucky Academy of Science is opposed to any attempt by legislative bodies to mandate specific content of science courses. The content of science courses should be determined by the standards of the scientific community. Science involves a continuing systematic inquiry into the manifold aspects of the biological and material world. It is based upon testable theories which may change with new data; it cannot include interpretations based on faith or religious dogma. As scientists, we object to attempts to equate "scientific creationism" or "intelligent design" with evolution as scientific explanations of events. Teaching faith-based models implies that these views are equivalent alternatives among scientists; doing so would be misleading to students. "Scientific creationism" and "intelligent design" are not equivalent to evolution. There is overwhelming acceptance by scientists of all disciplines that evolution (the descent of modern specifies of animals and plants from different ancestors that lived millions of years ago) is consistent with the weight of a vast amount of evidence. The understanding of the processes underlying evolution has provided the foundation upon which many of the tremendous advances in agriculture and medicine and theoretical biology have been built. Differences among scientists over questions of how evolution was accomplished do not obscure the basic agreement that evolution has occurred.
>
> Most people who subscribe to religious views have developed belief systems that are compatible with evolution. There is a widespread consensus among theologians that biblical accounts of creation are misunderstood if they are treated as literal scientific explanations. We fully respect the religious views of all persons but we object to attempts to require any religious teachings as science.
>
> We join the National Academy of Sciences, the American Association for the Advancement of Science, and the academies of science in many other states in calling for the rejection of attempts to require the teaching of "scientific creationism" and "intelligent design" as a scientific theory.
>
> It is further recommended that the Kentucky Academy of Science encourages its members and other professional scientific groups to give support and aid to those classroom teachers who present the subject matter of evolution fairly and encounter community objection. We also encourage administrators and individual teachers to oppose the inclusion of nonscientific concepts in the science classroom.

Passed KAS Annual Business Meeting, November 12, 1983.

And,

"A Resolution of the Kentucky Academy of Science In Regard to Omitting Evolution Terminology and Teaching in the Public Schools" (1999):

Whereas the Kentucky Academy of Science, founded in 1914, is an organization that encompasses all of the accepted scientific fields, and

Whereas the Scientific Method exemplifies that search for Scientific Understanding, and

Whereas this methodology has consistently provided the means of questioning dogma, authoritarianism, and deliberate deception, by championing the spirit of inquiry based on testing, analysis, honest review, criticism and counter criticism and designs for further testing, and,

Whereas the advancements of our understanding of the interconnection of the physical properties of our universe coupled with the life forms which together compose our biosphere clearly support that the evolutionary process has functioned and does function in the development, control, and survival of the earth's living beings, and

Whereas to deny the concepts of the known theoretical basis of the evolutionary process to the education arena of our public schools by avoiding or eliminating from the science curriculum any mention of the term evolution and evolutionary concepts would be an affront to an objective inquiry and the understanding of science,

Thereby be it resolved that the Kentucky Academy of Science, in the strongest and most determined ways possible, deplores the decision to substitute "change over time" for "evolution" in the state teaching standards, urges that the original wording be reinstated, and decries any attempt to remove the teaching of basic evolutionary theory or any scientific concept that may be tested and examined in concert with the basic scientific laws and principles that comprise the Scientific Method, and furthermore be it resolved that the public supported education systems of the Commonwealth be enhanced with complete support of seeking knowledge by every means possible commensurate with known principles of scientific theory, fact, and understanding.

Adopted by the KAS Governing Board November 6, 1999.
Passed unanimously by KAS membership November 6, 1999.

Both resolutions reviewed and reaffirmed by the KAS membership at the annual KAS business meeting on November 12, 2005.

The KAS also voted to endorse the following AAAS Board Resolution:

AAAS Board Resolution on Intelligent Design Theory

The contemporary theory of biological evolution is one of the most robust products of scientific inquiry. It is the foundation for research in many areas of biology as well as an essential element of science education. To become informed and responsible citizens in our contemporary technological world, students need to study the theories and empirical evidence central to current scientific understanding.

Over the past several years proponents of so-called "intelligent design theory," also known as ID, have challenged the accepted scientific theory of biological evolution. As part of this effort they have sought to introduce the teaching of "intelligent design theory" into the science curricula of the public schools. The movement presents "intelligent design theory" to the public as a theoretical innovation, supported by scientific evidence, that offers a more adequate explanation for the origin of the diversity of living organisms than the current scientifically accepted theory of evolution. In response to this effort, individual scientists and philosophers of science have provided substantive critiques of "intelligent design," demonstrating significant conceptual flaws in its formulation, a lack of credible scientific evidence, and misrepresentations of scientific facts.

Recognizing that the "intelligent design theory" represents a challenge to the quality of science education, the Board of Directors of the AAAS unanimously adopts the following resolution:

Whereas, ID proponents claim that contemporary evolutionary theory is incapable of explaining the origin of the diversity of living organisms;

Whereas, to date, the ID movement has failed to offer credible scientific evidence to support their claim that ID undermines the current scientifically accepted theory of evolution;

Whereas, the ID movement has not proposed a scientific means of testing its claims;

Therefore Be It Resolved, that the lack of scientific warrant for so-called "intelligent

design theory" makes it improper to include as a part of science education;

Therefore Be It Further Resolved, that AAAS urges citizens across the nation to oppose the establishment of policies that would permit the teaching of "intelligent design theory" as a part of the science curricula of the public schools;

Therefore Be It Further Resolved, that AAAS calls upon its members to assist those engaged in overseeing science education policy to understand the nature of science, the content of contemporary evolutionary theory and the inappropriateness of "intelligent design theory" as subject matter for science education;

Therefore Be It Further Resolved, that AAAS encourages its affiliated societies to endorse this resolution and to communicate their support to appropriate parties at the federal, state and local levels of the government.

Approved by the AAAS Board of Directors on November 18, 2002.

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Compiled by Ralph L. Thompson

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